

**TRANSLATING INSIGHTS FROM NORMAL, NEOPLASTIC
HEMATOPOIESIS AND MYELOID DISEASE PROGRESSION
INTO POTENTIAL CLINICAL APPLICATIONS**

by
Yiting Lim

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ABSTRACT

Certain cancers, especially those occurring in the hematopoietic system, can be identified by their cell of origin, and share many similar properties and signaling pathways to their normal counterparts. We use the hematopoietic system to interrogate the relationship between normal and neoplastic transformation. This work aims to better understand the molecular mechanisms of disease transformation in hematologic malignancies and translate basic biology findings into potential clinical applications.

To examine how normal hematopoiesis is perturbed, we investigated how low dose-rate radiation affected hematopoietic cells in a whole animal model, and uncover mechanisms to mitigate this adverse effect. Although the effects of acute radiation exposure have been well studied for many years, it is likely that widespread life-threatening radiation incidents will occur in the form of lethal doses delivered at relatively low rates over protracted time periods. We show that damage to hematopoietic progenitors and hematopoietic failure is a consequence of such radiation exposure. In addition, the anti-malaria agent chloroquine can protect these hematopoietic progenitors by activating ATM, a key player in the DNA damage response pathway, thus enhancing overall survival. We provide a mechanistic explanation and potentially viable prophylactic therapy for protracted low dose-rate radiation induced death.

The Hedgehog signaling pathway is highly conserved and important for development. Though its role in normal hematopoiesis is controversial, it does not impact normal adult hematopoiesis. Hedgehog signaling is aberrantly regulated in many cancers, including several hematopoietic malignancies. We found Hedgehog signaling to be upregulated in human secondary leukemias.

We hypothesize that Hedgehog signaling plays a role in disease progression and generated a novel transgenic mouse model of myeloid disease progression. Conditional activation of Hedgehog signaling dramatically accelerated disease progression from a chronic myeloproliferative disorder initiated by an activating mutation in the FLT3 tyrosine kinase receptor to rapidly fatal acute myeloid leukemia. Mice harboring both the *FLT3-ITD* (a commonly found mutation in adult AML) and *SmoM2*, (a constitutively active form of Smoothened) alleles died rapidly from AML caused by increased myeloid progenitor proliferation. Pharmacologically inhibiting both Hedgehog and FLT3 pathways synergistically reduced leukemic growth and improved overall survival compared to targeting either pathway alone.

Thesis Readers:

William H. Matsui, M.D (Thesis Advisor)

Professor of Oncology, Johns Hopkins University School of Medicine

Donald Small, M.D/Ph.D (Thesis Committee Chair)

Kyle Haydock Professor of Oncology, Johns Hopkins University School of Medicine

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LIST OF ABBREVIATIONS

AML	Acute myeloid leukemia
ATM	Ataxia telegectasia mutated
B-ALL	B-cell acute lymphocytic leukemia
BCC	Basal cell carcinoma
BrdU	Bromodeoxyuridine
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
Ci	Cubitus Interruptus
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
Cos2	Costal2
Dhh	Desert hedgehog
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	Food and drug administration
FLT3-ITD	Fms-like tyrosine kinase 3 - internal tandem duplication
FLT3-TKD	Fms-like tyrosine kinase 3 – tyrosine kinase domain
FLT3-WT	Fms-like tyrosine kinase 3 – wild type
FOXO	Forkhead box O class

Fu	Fused
GMP	Granulocyte–macrophage progenitor
GSEA	Gene set enrichment analysis
Hh	Hedgehog
HPE	Holoprosencephaly
HSC	Hematopoietic stem cell
hTERT	human telomerase reverse transcriptase
Ihh	Indian hedgehog
JM	Juxtamembrane
KLF6	Krüppel-like factor 6
KSL	c-kit-positive, Sca-1-positive, lineage marker-negative
LDR	Low dose rate
LT-HSC	Long-term hematopoietic stem cell
MB	Medulloblastoma
MDS	Myelodysplastic syndrome
MEK	Mitogen activated protein kinase kinase
MEP	megakaryocyte erythroid progenitor
MLL-AF9	Mixed-lineage leukemia – AF9
MPP	Multipotent progenitor
Mx1	Myxovirus-resistance 1
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NPM	Nucleophosmin
pATM	Phosphorylated ataxia telegectasia mutated
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PML-RAR α	Promyelocytic leukemia – retinoic acid receptor-alpha
Poly(I:C)	Polyinosinic:polycytidylic acid
Ptch	Patched
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
R26	Rosa 26
RBC	Red blood cell
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Shh	Sonic hedgehog
Smo	Smoothened
SP	Side population
ST-HSC	Short-term hematopoietic stem cell
SuFu	Suppressor of Fused
T-ALL	T-cell acute lymphocytic leukemia
TBI	Total body irradiation
TGF β	Transforming growth factor beta
YFP	Yellow fluorescent protein

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CHAPTER 1: INTRODUCTION

Cancer – biology, clinical advances, challenges

Cancer is characterized by the uncontrolled growth and spread of abnormal cells, and is the second most common cause of death in the US.¹ There have been significant efforts and progress at better diagnoses and more effective treatment in an attempt to eradicate cancer as a major cause of death, including understanding the biology of the disease. However, several challenges still remain. The biology of cancer is inherently complex – there are multiple changes and cellular interactions within a cell and in its surroundings that contribute to its transformation to an abnormal state. There is also significant heterogeneity in disease across different organs and tissue systems, and a lack of suitable model organisms to accurately understand human disease. Furthermore, the lengthy drug development and approval process also pose as obstacles to getting new therapies to patients.

Aims of thesis

My goal is to investigate how normal cells transform to become abnormal in the development of cancer, and how we can interrupt this process to improve on treating the disease. I am interested in studying this in the context of normal hematopoiesis and hematologic malignancies because hematopoietic development is a clearly delineated process, where cells can be identified and isolated at different stages of development and disease. Additionally, by investigating mechanisms of disease transformation in this system, we can gain insights into how normal hematopoietic cells become abnormal resulting in aggressive disease, and translate these findings into potential clinical applications. The next chapter summarizes the tools and technology we have to study normal and malignant hematopoiesis both *in vitro* and *in vivo*. Chapter 3 shows how these tools are used to investigate perturbations to normal hematopoiesis during low dose-rate radiation exposure, and describes how the anti-malaria drug chloroquine can play a unique role in mitigating this fatal damage to normal hematopoiesis. Chapter 4 describes a novel mouse

model of myeloid disease progression and sheds light on the role of a developmental signaling pathway in disease. Chapter 5 shows the clinical relevance of our findings in Chapter 4, and provides rationale for translating these pre-clinical findings into potential clinical applications.

Hematopoiesis and Hematologic malignancies

Hematopoiesis is defined as the formation of all types of blood cells from hematopoietic stem cells (HSCs) in the bone marrow.² These cells include erythrocytes that provide oxygen carrying capacity, platelets that are crucial for hemostasis through blood clot formation, and leukocytes that maintain host defense to infections. An inability to maintain the production of these circulating cells through regulating the proliferation, differentiation and maturation of immature progenitor cells will put an individual at risk for hematologic disorders. HSCs maintain blood production in the adult by maturing into multipotent progenitors (MPP) that subsequently differentiate into lineage-committed common myeloid progenitors (CMP) and common lymphoid progenitors (CLP) that eventually produce mature blood cells.^{2, 3} This hierarchical production of blood cells from their progenitors is a well delineated process; we currently have the technology to identify and isolate hematopoietic stem and progenitor cells at various stages of development. Furthermore, HSCs undergo self-renewal that allows the maintenance of blood production over the lifetime of the organism. In the development of leukemia, many of the features of normal hematopoiesis are retained. Normal HSCs acquire genetic mutations and leukemogenic events that lead to malignant clonal expansion. These dominant clonal populations retain the hierarchical organization found in normal hematopoiesis, however the leukemic descendants retain a primitive phenotype. Signaling pathways and transcriptional regulators that govern the hierarchical production of blood cells may also be present in hematopoietic malignancies. Moreover, the clinical importance of hematopoiesis is undeniable – benign hematologic and bone marrow failure syndromes, leukemia, lymphoma, myeloma and myelodysplastic syndromes

all originate from hematopoietic cells. HSCs also serve as a source of marrow transplantations to treat both malignant and non-malignant diseases.

CHAPTER 2: METHODS TO STUDY NORMAL AND MALIGNANT HEMATOPOIESIS

Identification of cell types

By morphology

Several techniques are available to distinguish the different types of blood cells according to their different biological and functional characteristics. Firstly, different types of blood cells can be identified by their distinct morphology. This is done by staining blood cells and bone marrow aspirates with the Wright-Giemsa stain, and examining them under a light microscope. The hematoxylin eosin stain is also used on tissue sections such as the bone marrow and spleen to distinguish different cell types. Other cytochemical stains that are more specific for certain cell types, such as the myeloperoxidase stain and the chloro-acetate esterase, are also routinely used in the diagnosis and classification of acute leukemias.⁴

By cell surface markers

Hematopoietic cells can also be identified and isolated based on the unique cell surface molecule expression on these cells, and has been greatly facilitated by technological advances in monoclonal antibody availability and flow cytometry. HSCs can be readily identified by multi-parameter flow cytometry analysis. Although different groups have developed different antibody combination schemes to purify HSCs from mouse bone marrow, they are all driven by the same goal of isolating the highest yield of long-term, self-renewing, multi-lineage reconstituting HSCs. Almost all of these strategies revolve around the positive selection for the markers cKit and Sca1, and negative selection for markers of mature hematopoietic cell lineages (typically B220, CD4, CD8, Gr1, Mac1 and Ter119). This cKit⁺ Lin⁻ Sca1⁺ (KSL) phenotype greatly enriches for the ability of these cells to reconstitute hematopoietic activity, but also contains progenitor cells in addition to long-term HSCs. Other strategies to identify HSCs include CD34 and FLT3 with KSL, CD150 and CD48 with or without KSL, as well as the Hoechst-effluxing side population (SP).⁵⁻⁷ The analysis of short-term HSCs and hematopoietic progenitor cells include phenotypes

to identify common lymphoid progenitors (CLP) and common myeloid progenitors (CMP), as well as their downstream progeny including pro-T and pro-B cells in the lymphoid lineage and megakaryocyte-erythrocyte progenitors (MEP) and granulocyte-macrophage progenitors (GMPs) in the myeloid lineage. CLPs can be identified in whole bone marrow using KSL with IL7 α . The CMP population is identified in the Sca-negative portion of the KSL compartment, and is also negative for IL7 α . This fraction can be further subdivided into CMP (CD34⁺ CD16/32⁻), MEP (CD34⁻ CD16/32⁻) and GMP (CD34⁺ CD16/32⁺). Furthermore, these different hematopoietic stem and progenitor cell populations can be purified by cell sorting.⁷ Mature blood cells can also be identified by their cell surface phenotype in peripheral blood. B220 identifies the B-lymphocytes, CD3 and CD4 identify the T-lymphocytes, and Mac1 and Gr1 identify the myeloid cell lineage in peripheral blood. Healthy blood should contain all the mature B, T-cell and myeloid lineages in order to carry out its normal functions.

By function

Hematopoietic stem and progenitor cells can also be identified according to their function. HSC function is generally monitored according to their functional contribution to generating blood cells. The best method to do this *in vivo* is to transplant test cells into recipient animals in which their hematopoietic system has been ablated by radiation. The function of the transplanted bone marrow cells are then assessed by determining the level of chimerism of the recipients by sampling their peripheral blood samples at various timepoints after the transplant. The recipient and donor mice have different alleles of the CD45 antigen (donor cells are typically isolated from CD45.2 mice with the standard C57BL/6 allele, while recipient mice are usually the CD45.1 congenic strain), which can be distinguished using specific monoclonal antibodies against CD45.1 and CD45.2. The function of transplanted HSCs can be assessed in terms of overall contribution to the recipient's peripheral blood chimerism by engraftment of donor cells, as well as the types of hematopoietic cells generated from the transplanted HSCs by lineage analysis of

peripheral blood. Antibodies are used to track the three main mature blood lineages in peripheral blood - myeloid cells (Gr-1+, Mac-1+), B cells (B220+) and T cells (CD4+, CD8+). By comparing the distribution of cell types formed from transplanted test cells to wild-type controls, we can determine if the transplanted cells have functional bias for making particular lineages. Timing of hematopoietic constitution can also be used to determine function of hematopoietic stem and progenitor cells. After 4-weeks, the peripheral blood cells formed from input test cells can be the progeny of short-term HSCs or long-lived progenitors. However, the only test cells that can self-renew for 16-weeks post-transplant are long-term HSCs; thus, the peripheral blood components generated from test cells after this time period are the progeny of these cells. The myeloid progenitor function of bone marrow cells can also be functionally assessed *in vitro* by plating these cells in semi-solid media supplemented with cytokines to promote the growth of these myeloid progenitor colonies. Colonies can be scored according to their distinct morphology.

Mouse models of hematopoiesis and leukemia

The mouse has been used extensively to study hematopoiesis and model human hematologic malignancies to investigate the basic genetic and biochemical components of malignant behavior. Furthermore, mice can also be used in the preclinical evaluation of novel therapies.⁸ Controlled experiments that are difficult or impossible in humans can be performed in mice, and unlike patients, mice can be designed to have both a defined genotype and congenic siblings that serve as controls.

Several distinct methods can be used to introduce oncogenic mutations into the murine hematopoietic system. In conventional transgenic models, an oncogene is integrated at a random site in the genome. Retroviral transduction is a rapid method for generating series of genetically

related leukemias. In some cases, proviral insertion can be deliberately exploited to generate leukemias by insertion mutagenesis.⁹ While these systems have proven quite valuable, they suffer from poor control over oncogene copy number and expression pattern due to integration effects. Conditional gene targeting addresses these concerns by modifying the endogenous locus of a proto-oncogene or tumor suppressor gene and allows a mutation be induced at a specific time and/or lineage. To date, this approach provides the most accurate genetic model of oncogenic mutations. Xenograft models where human leukemia cells are engrafted into immunocompromised mice have also been generated. Although this system lacks the ability to be genetically manipulated, the main advantage is that actual human leukemia cells are used.

Limitations and advantages

Although mouse models have become an indispensable tool in hematopoiesis and cancer research, they have to be used with discretion. Firstly, the genetically engineered oncogene may not fully recapitulate what is actually happening in the patient, and may not represent initiating events in human hematologic cancers. Secondly, differences in pharmacology and toxicity in the mouse may also be challenging in translating preclinical drug studies into patients. Nonetheless, there are extensive similarities between human and mouse hematopoiesis. There is also an amalgam of techniques to assess cell biology in both normal and diseased states of hematopoiesis in the mouse. Generous amounts of hematopoietic cells can be easily obtained from the tissues such as the bone marrow, spleen and peripheral blood, and characterized. Hematopoietic malignancies can often times be transplanted into naïve hosts to propagate the disease in these recipients. This not only allows the system and diseased organisms to be scaled up, it also allows for the study of the self-renewal function of diseased donor cells. Mouse bone marrow transplantation has been the gold standard for the functional characterization of HSC activity. It is also a great *in vivo* model for bone marrow transplantation studies in humans.

CHAPTER 3: PERTURBATIONS TO NORMAL HEMATOPOIESIS USING LOW DOSE-RATE RADIATION, EFFECT OF CHLOROQUINE¹

¹ Reprinted from Int J Radiat Oncol Biol Phys, Vol 84(3), Lim Y, Hedayati M, Merchant AA, Zhang Y, Yu HH, Kastan MB, Matsui W, Dewese TL., Chloroquine improves survival and hematopoietic recovery after lethal low-dose-rate radiation, 800-806, Copyright (2012), with permission from Elsevier.

Introduction:

The biologic effects of ionizing radiation have been studied for over a century, but it was not until the deployment of the atomic bomb in 1945 that the clinical manifestations of total body irradiation (TBI) were fully realized. Following acute radiation exposure, hematopoietic defects in particular are observed and are an important cause of death. This phenomenon has been attributed to the depletion of rapidly cycling and highly sensitive hematopoietic progenitors leading to myelosuppression.¹⁰⁻¹³ Radioprotective agents that allow survival of these short-lived myeloerythroid progenitor cells to replenish mature blood cells may prevent mortality from radiation-induced hematopoietic injury.

Radiation dose-rate is an important factor determining the extent of cellular toxicity during radiation exposure.¹⁴ Previous studies have found that the delivery of radiation doses at low dose-rates (LDR) may result in greater or lesser amounts of cell killing *in vitro* compared to equivalent doses delivered at higher dose-rates.^{15, 16} At specific dose rates (> 5 cGy/hr), greater cell survival is observed compared to equivalent doses applied at higher dose rates, presumably because of ongoing repair of radiation-induced injury during the protracted exposure. This has been termed the “low dose rate effect”.^{17, 18} In contrast, dose rates of < 2 cGy – 1 Gy/hr can actually increase cell death relative to the same total dose delivered in a rapid, acute fashion and has been termed the “inverse dose rate effect”.^{15, 19-21} This has been hypothesized to be a result of alterations in cell cycle or reduced repair of radiation-induced DNA injury.^{21, 22} One of the key proteins involved in the response of mammalian cells to radiation-induced injury is ataxia telegectasia mutated (ATM) that is activated by autophosphorylation following DNA damage.²³ Once activated, ATM subsequently phosphorylates other key proteins involved in the repair of DNA damage.²⁴ The anti-malarial agent, chloroquine, has been shown to activate ATM without inducing DNA injury presumably by altering chromatin structure.^{23, 25} Intriguingly, ATM is not activated by LDR

radiation, but the addition of chloroquine to cancer cells growing *in vitro* prior to LDR radiation exposure activates ATM and subsequently reduces cell death from LDR.²⁶ We examined whether chloroquine could similarly act as a radioprotective agent *in vivo* and treated mice with chloroquine prior to LDR radiation exposure. We found that chloroquine improved survival of the animals by enhancing the recovery of hematopoietic progenitors responsible for early engraftment. Furthermore, the effects of chloroquine were dependent on the activation of ATM protein. These data serve to expand knowledge regarding the role of ATM in radiation injury in mammals and highlight the possibility that drugs like chloroquine may serve as modulators of radiation-induced injury.

Methods:

Cell proliferation assay

Human fibroblast cells obtained from an *ATM*^{-/-} patient were immortalized using *hTERT* (GM05823-*hTERT ATM*^{-/-}). Immortalized wild-type human fibroblast cells (HFF-*hTERT ATM*^{+/+}) were used as control. Cells were cultured in DMEM with 10% FBS and treated with 48µg/ml chloroquine for 4h, then washed with PBS and cultured in 10 ml of fresh medium. Flasks were gassed with 5% CO₂, sealed and kept in the low dose-rate irradiator for 42.5hrs at 37°C for a total radiation exposure of 4 Gy at a rate of 9.4 cGy/hr. Cell proliferation was assessed using Cell titer Blue (Promega, Madison, WI). Fluorescence was measured using a microplate system (SpectraMax M2, Molecular devices). 1000 cells/well were used for GM5823-*hTERT (ATM*^{-/-}) and 400 cells/well were used for HFF-*hTERT (ATM*^{+/+}).

Mice

Male C57BL/6-CD45.2 mice (Harlan Laboratories, Indianapolis, IN) were used as bone marrow donors and female C57BL/6-CD45.1 mice (National Cancer Institute) as transplant recipients. Male C57BL/6 *Atm* null mice were used in experiments to determine the influence of ATM in total body radiation-induced death (St. Jude Children's Research Hospital, Memphis, TN).²⁷ *Atm* status was confirmed by PCR of mouse genomic DNA. All the mice were used at 4 weeks of age, and housed under specific pathogen-free conditions in an accredited facility at the Johns Hopkins Medical Institutions. All experiments were conducted using protocols approved by the Johns Hopkins Institutional Animal Care and Use Committee (IACUC).

Radiation exposure and chloroquine administration

Wild type C57BL/6 donor mice were exposed to 12.8 Gy of TBI in a custom-built low dose-rate irradiator at 9.4 cGy/hr for 5.5 days (total of 136 hrs).¹⁶ *Atm*^{-/-} mice were similarly treated but received a total of 9 Gy, given their greater radiosensitivity.²⁷ Bone marrow recipient mice were conditioned with 10 Gy given as two 5 Gy fractions 4hrs apart prior to bone marrow transplantation. Chloroquine (Sigma-Aldrich, St. Louis, MO) was dissolved in PBS, filter-sterilized and injected intraperitoneally (0.0594 mg per 17g body weight) in two doses administered 24hrs and 4hrs prior to LDR radiation exposure.

Bone marrow transplantation

Donor mice were sacrificed by cervical dislocation immediately after LDR radiation exposure. Bone marrow cell suspensions were prepared by crushing the tibia, fibula and vertebral bones with a mortar and pestle in sterile PBS followed by passage through a 70-µm filter. Bone marrow cellularity was determined using a Coulter counter. Whole bone marrow cell suspensions in PBS (300 µl total volume) were injected via the retro-orbital venous sinus into lethally irradiated recipients.

Complete peripheral blood cell count

Peripheral blood (50 µl) was collected from murine retroocular vessels using heparinized capillary tubes and complete blood cell counts were obtained using a Hemavet950 Hematology system (Drew Scientific, Oxford, CT).

In vitro methycellulose colony forming assay

Whole bone marrow cell suspensions were cultured in semi-solid methylcellulose medium (M3434, Stem Cell Technologies, Vancouver, Canada) supplemented with recombinant murine SCF (50 ng/mL), IL-3 (10 ng/mL), IL-6 (10 ng/mL), GM-CSF (10 ng/mL) and EPO (3 U/mL).

Cells were incubated at 37°C with 5% CO₂, and total number of colonies were counted after 10 days using an inverted microscope.

In vivo progenitor assay

1 X 10⁷ whole bone marrow cells were harvested from LDR irradiated mice, mixed with 2 X 10⁵ congenic CD45.1 un-irradiated bone marrow cells and transplanted into lethally irradiated C57BL/6 CD45.1 recipients. To assess donor engraftment, peripheral blood (50 µl) was drawn via retro-orbital bleeding into heparinized capillary tubes and stained with anti-mouse CD45.1-FITC and CD45.2-PE antibodies (eBioscience, San Diego, CA), followed by flow cytometry.

Lineage depletion and FACS analysis

Whole bone marrow cell suspensions were stained with a mixture of purified biotin conjugated monoclonal antibodies recognizing mouse Ter-119, CD3e, B220 and Gr-1 (eBioscience, San Diego, CA). Cells positive for lineage markers were partially removed by magnetic bead depletion on a LD column with Anti-Biotin MicroBeads, mouse IgG1 isotype (Miltenyi Biotech, Auburn, CA). The remaining lineage depleted cells were collected in the flow through from the magnetic columns and treated with 35 µg/mL of chloroquine (Sigma) for 2.5hrs at 37°C and 5% CO₂. To detect phosphor-ATM expression, the lineage depleted cells were washed with PBS, fixed in 2% formaldehyde for 10 minutes at room temperature, permeabilized with a mixture of cold 50% methanol and 50% acetone and blocked with 2% FBS in PBS overnight. Cells were then stained with anti-pATM-PE (clone 10H11.E12, Millipore, Billerica, MA), which recognizes phosphorylated Ser-1981, and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and FlowJoTM software (TreeStar, Ashland, OR).

Western blots

Whole bone marrow cells from chloroquine treated or untreated mice were analyzed by western blot according to standard protocols. Briefly total cell lysates were prepared from about 5×10^7 whole bone marrow cells. About 200 μg of the total cell lysate was loaded per lane. Total p53 was detected by using anti p53 (EMD Chemicals, Gibbstown, NJ) and phosphorylated p53 was detected by using anti mouse phospho-p53 (S18) antibody (R&D SYSTEM, Minneapolis, MN) at final concentration of 2 $\mu\text{g}/\text{ml}$.

Statistical analysis

Survival was calculated using Kaplan Meier analysis and a log rank test. Results are presented as the mean \pm standard error of the mean. Comparisons between 2 groups, chloroquine treated and untreated, were performed using a 2-tailed, un-paired Student t-test. P values <0.05 were considered statistically significant.

Results:

ATM is necessary for the radioprotective effect of chloroquine

Chloroquine has long been utilized as an anti-malarial agent. It is also known to induce the activation of ATM and has been shown to abrogate the enhanced cytotoxicity of LDR radiation *in vitro*.²⁶ To further examine whether the radioprotective effect of chloroquine is dependent on ATM, we analyzed the effect of chloroquine on *hTERT* immortalized human fibroblasts derived from normal donors or patients with ataxia-telangiectasia that lack *ATM* activity. Treatment of normal fibroblasts with chloroquine prior to LDR radiation improved cell viability, similar to our previous *in vitro* findings using cancer cells (Figure 3.1A).¹⁵ In contrast, chloroquine failed to protect *ATM* deficient fibroblasts from LDR irradiation (Figure 3.1B). Compared to untreated animals, mice pre-treated with two doses of chloroquine demonstrated significantly improved survival rates (Figure 3.1C, 21% vs. 79%; $P < 0.05$). We also examined whether ATM was specifically required for chloroquine-mediated protection from LDR radiation *in vivo* and treated *Atm* null transgenic mice with or without chloroquine prior to LDR radiation exposure. In contrast to wild-type mice (Figure 3.1C), we detected no significant differences in survival between chloroquine treated and untreated *Atm* null mice (Figure 1D, 61.5% vs. 69.2% $P = 0.86$). Therefore, both the *in vitro* and *in vivo* radioprotective effects of chloroquine require intact ATM.

Figure 3.1: Radioprotection by chloroquine requires ATM in vitro and in vivo.

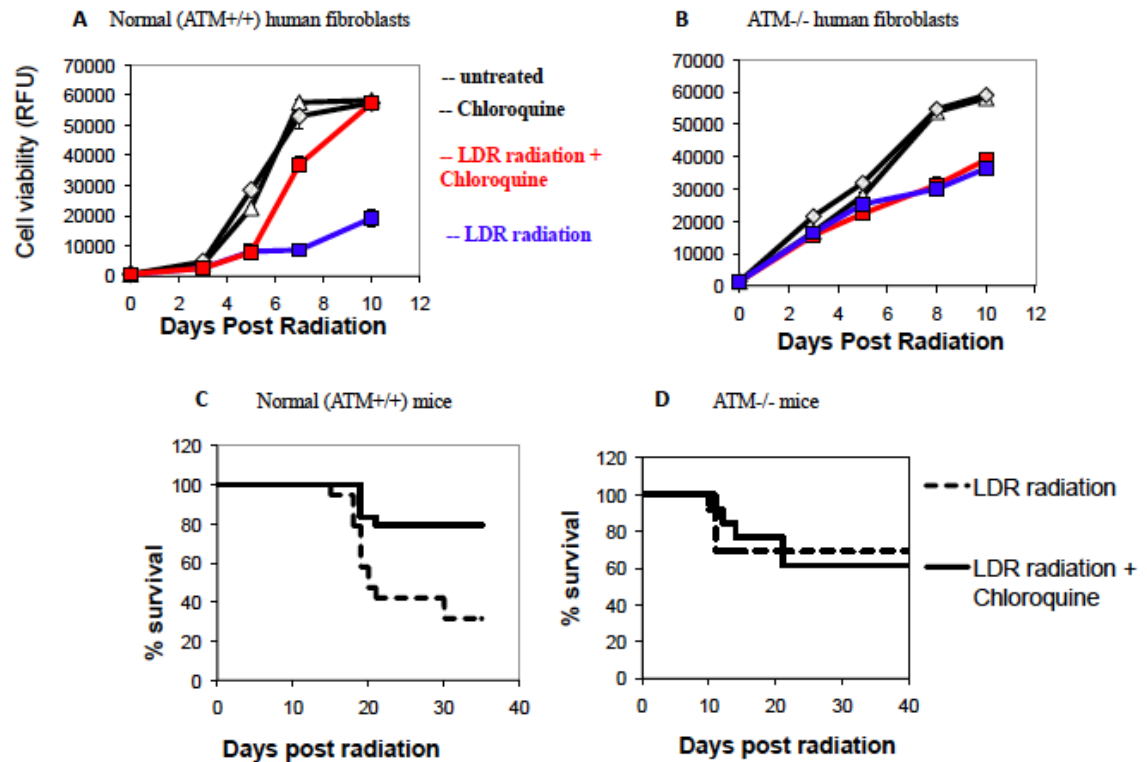
(A) Effect of chloroquine on the proliferation of wild type *ATM*^{+/+} human fibroblast cell line (HFF-*hTERT*) in the presence of *ATM* following 4Gy of ionizing radiation at LDR radiation exposure.

(B) Effect of chloroquine treatment on *ATM* deficient fibroblast cells (GM5823 *hTERT*).

(C) Kaplan-Meier survival analysis of wild-type C57BL/6 mice exposed to LDR radiation. Mice were untreated (dashed line, n=29), or pre-treated with chloroquine before LDR radiation exposure (solid line, n=24; *P< .05)

(D) Kaplan-Meier survival analysis of *Atm*^{-/-} mice treated with or without chloroquine prior to LDR radiation (P=0.865; n=13).

Figure 3.1



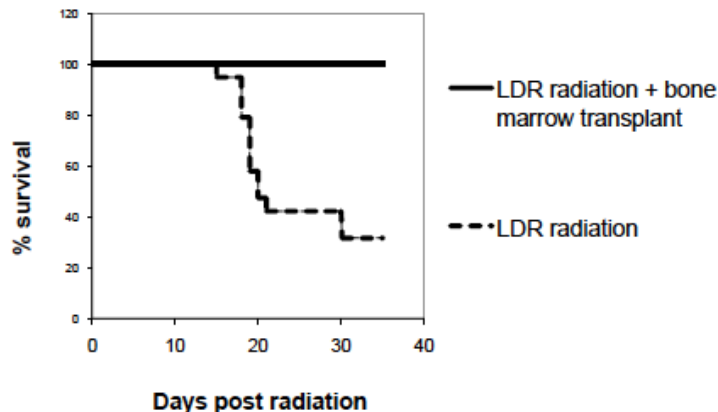
Low dose-rate radiation induces lethal bone marrow failure

The ability of bone marrow transplantation to rescue acutely irradiated mice indicates that death results from hematopoietic failure.²⁸ Since radiation exposure over a protracted time period can enhance cell death *in vitro*, we examined whether bone marrow failure was similarly responsible for death following lethal irradiation delivered at a low dose-rate. We initially exposed C57BL/6 mice to 12.8 Gy of TBI at a rate of 9.4 cGy/hr for 136 hours and found that only 6 of 29 (21%) of animals survived by day 35. However, all mice (n=10) receiving whole bone marrow (1×10^6 cells) immediately after LDR radiation survived for greater than 35 days (Figure 3.2, $P < 0.05$). Full necropsies following treatment with LDR radiation alone failed to demonstrate significant damage to the lungs or gastrointestinal tract (data not shown), suggesting that impaired hematopoiesis is a primary cause of death following exposure to LDR radiation in these animals.

Figure 3.2: LDR radiation causes bone marrow failure

Kaplan-Meier survival analysis of wild-type C57BL/6 mice exposed to LDR radiation. Mice were untreated (dashed line, n=29), or transplanted with 1×10^6 whole bone marrow cells (solid line, n=10) ($P < .05$)

Figure 3.2



Chloroquine protects hematopoietic progenitors from LDR radiation

Bone marrow rescue following acute lethal radiation is primarily mediated by committed myeloid progenitors.²⁹ We examined the effects of chloroquine treatment on myeloid progenitor cell function by plating whole bone marrow cells from LDR irradiated mice in methylcellulose and quantifying colony-formation. LDR radiation alone decreased the total number of bone marrow cells by 5-fold (data not shown) and colony formation compared to un-irradiated samples (Figure 3.3A, 0.76 vs. 13). However, compared to untreated animals, chloroquine treatment significantly improved the recovery of total myeloid colony forming unit (CFC) following radiation (Figure 3.3A, 0.76 vs. 1.57; $P < .05$). Furthermore, this effect was primarily due to protection from radiation injury as no significant differences in myeloid CFC were observed in un-irradiated mice treated with or without chloroquine (Figure 3.3A; $P=0.30$).

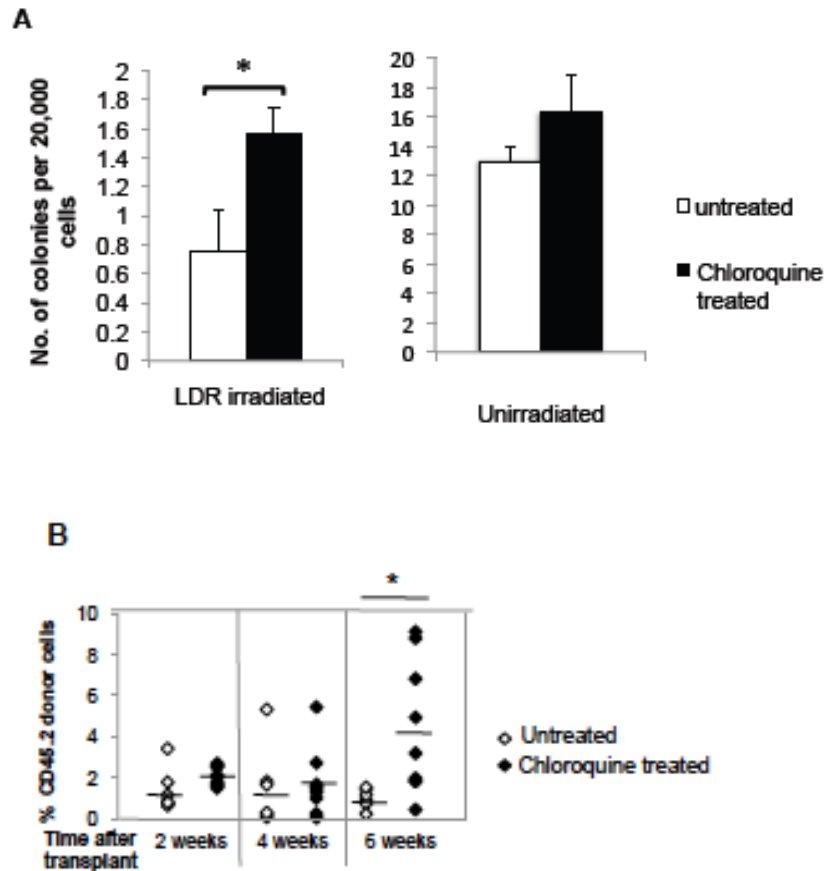
We also examined the effects of chloroquine on the *in vivo* recovery of myeloid progenitors and transplanted 1×10^7 whole bone marrow cells from LDR irradiated C56BL/6 CD45.2 donor mice into congenic C57BL/6 CD45.1 recipient mice. Since myeloid progenitors are responsible for early engraftment, we quantified the peripheral blood chimerism by flow cytometry starting 2 weeks following bone marrow transplantation. In mice receiving bone marrow from LDR irradiated mice pretreated with chloroquine, the frequency of peripheral blood CD45.2 donor cells was significantly increased compared to untreated mice by 6 weeks post transplant (Figure 3.3B, 4.33% vs. 1.00%; $P=0.015$). In comparison, chloroquine treatment did not impact long-term engraftment (> 12 weeks) of LDR radiated bone marrow (data not shown), suggesting little impact on long-term hematopoietic stem cells. Therefore, the enhanced survival of LDR irradiated mice treated with chloroquine is primarily mediated through the protection of hematopoietic progenitors.

Figure 3.3: Chloroquine protects hematopoietic progenitors from LDR radiation in vitro and in vivo.

(A) Hematopoietic progenitor colony formation *in vitro*. Chloroquine pre-treated or untreated whole bone marrow cells from LDR irradiated or non-irradiated mice were assessed for colony formation after 10 days in methylcellulose ($P < 0.05$; $n = 3$).

(B) *In vivo* engraftment analysis of mice transplanted with 1×10^7 whole bone marrow cells following LDR irradiated with or without chloroquine treatment. Peripheral blood was stained with CD45.1 and CD45.2 antibodies to assess chimerism. Each data point is representative of one mouse.

Figure 3.3



Chloroquine activates ATM in mouse hematopoietic progenitor cells

Chloroquine binds to DNA by intercalating between bases and alters the internucleosomal DNA helical twist and chromatin structure *in vitro* and *in vivo*.^{30, 31} Changes in chromatin structure subsequently induce the activation of ATM, a key mediator of the DNA damage response.²³ Chloroquine has also been shown to activate ATM and its downstream effectors *in vivo*.³² Therefore, chloroquine is thought to protect cells from LDR radiation by activating ATM and subsequent downstream effectors. Since chloroquine improved the recovery of myeloid progenitors following lethal LDR radiation, we examined whether it activated ATM in these cells. We isolated lineage-depleted mouse bone marrow cells and treated them with chloroquine (35 µg/ml for 2 hours) and examined the activation status of ATM by flow cytometry. Compared to untreated cells, chloroquine significantly increased the expression of activated phosphorylated ATM by approximately 2.5 fold (Figures 3.4A and 3.4B; $P < .05$). We also examined the expression of phosphorylated ATM following *in vivo* treatment with chloroquine; however, we did not detect significant ATM activation in lineage negative bone marrow cells likely due to the cellular processing required to isolate hematopoietic progenitors. We could not detect phosphorylated ATM by standard western blotting techniques of whole bone marrow obtained from treated mice. The difficulty of detecting phosphorylated ATM in mouse tissue has been reported.³² Previous studies have used the activation status of p53, a downstream target of activated ATM, as a surrogate for *in vivo* ATM phosphorylation and have shown increased p53 phosphorylation in tissues from chloroquine treated mice.³² Therefore, we examined the level and activation status of p53 in bone marrow cells obtained from mice treated with chloroquine. Chloroquine treatment increased the levels of both total and phosphorylated p53 (Ser 18) as detected via western blot analysis, consistent with canonical ATM activation (Figure 3.4C). Taken together these results support *in vivo* activation of ATM in bone marrow cells by chloroquine.

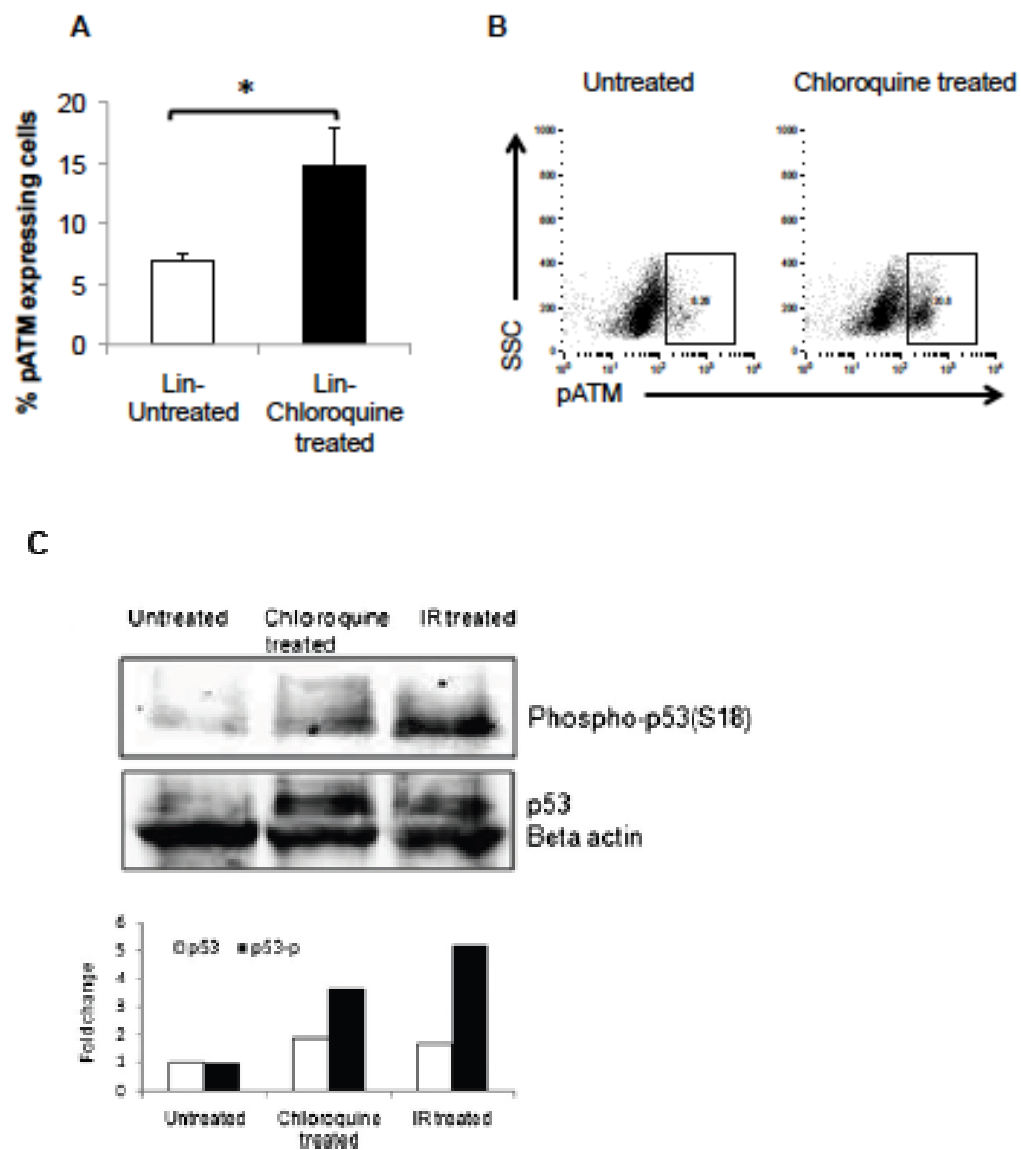
Figure 3.4: Chloroquine activates ATM in mouse hematopoietic progenitor cells.

(A) Lineage depleted mouse bone marrow cells were treated with chloroquine and analyzed by flow cytometry for pATM expression. Quantitation of phosphorylated ATM expression by flow cytometry analysis ($P < 0.05$; $n = 3$).

(B) Representative dot plots gated on forward and side scatter followed by pATM expression. Untreated cells were stained as a control.

(C) Western blot of p53 expression in mouse bone marrow cells used as surrogate for ATM activation. Radiation treated mouse used as positive control for p53 phosphorylation received 5 Gy of acute TBI. Graph of quantitative fold change of total p53 and p53-p normalized to beta-actin is included.

Figure 3.4



Discussion:

Protracted LDR radiation exposures arising from nuclear accidents and terrorism events may pose serious threats to public health. While it is well known that deaths from hematopoietic defects occur following acute radiation exposure, we found that bone marrow transplantation can similarly rescue mice following LDR radiation. Therefore, bone marrow failure is a primary cause of death, and chloroquine can protect myeloid progenitors from LDR radiation resulting in improved overall survival.

Despite the potentially devastating consequences of LDR radiation exposure, few protective agents are currently available.³³ Agents that confer radioprotection can be generally classified into three categories: sulfhydryl compounds, antioxidants and receptor-mediated agents such as lipids, cytokines and growth factors.³⁴ Cysteine, a sulfur containing amino acid, was first shown to protect rats from lethal x-radiation.³⁵ Similarly, nutraceuticals, such as *N*-acetyl cysteine or genistein, have been found to provide a hematopoietic radioprotective effect in animal models by protecting cells from oxidative stress.³⁶ Hematopoietic growth factors and cytokines have also been shown to improve animal survival after TBI.³⁷ These agents have been proposed as protective agents following radiation exposure during cancer therapy and or in the event of a nuclear accident. However, many of these agents lack the properties of robust activity, prolonged efficacy, broad specificity and minimal toxicity required to effectively protect a large population during a radiological emergency. There is great potential for chloroquine to serve as a potential radioprotective agent following LDR radiation exposure since it is a cheap, widely available drug with minimal side effects.

The precise molecular mechanisms by which chloroquine protects cells from LDR radiation are unknown, but some radioprotective agents, such as aminothiols, are thought to

structurally stabilize DNA and decrease the rate of DNA replication.³⁸ Similar to aminothiols, chloroquine binds to DNA and its intercalation between DNA bases results in the structural modification of chromatin.³⁹ This alteration in chromatin structure has been associated with the activation of ATM in cell lines.²³ Likewise, we found that chloroquine activates ATM in hematopoietic progenitors, and several potential mechanisms may confer radioresistance. The well-recognized role of ATM in regulating the DNA damage response suggests that it may activate DNA repair pathways that otherwise fail to be induced by LDR radiation.⁴⁰ ATM also regulates the cellular response to oxidative stress and reactive oxygen species (ROS) that are increased following radiation exposure.⁴¹ In the hematopoietic system, the loss of ATM results in impaired hematopoietic stem cell function that is associated with increased levels of ROS.^{42, 43} Although we did not observe differences in the long-term engraftment of bone marrow derived from chloroquine treated animals indicative of effects on hematopoietic stem cells, it is possible that ATM activation enhances cytoprotective mechanisms within progenitors that abrogate the damaging effects of increased levels of ROS following exposure to LDR radiation.⁴⁴ Recently, ATM has also been found to play a role in regulating cellular metabolism and autophagy in response to increased ROS levels by activating *TSC2* and the LKB1/AMPK pathway to ultimately repress mTORC1 signaling.⁴⁵⁻⁴⁷ Several studies have demonstrated that the loss of *Lkb1* severely impairs hematopoiesis in mice primarily by decreasing mitochondrial function.⁴⁸⁻⁵⁰ Therefore, it is also possible that chloroquine-induced activation of ATM and subsequent LKB1/AMPK signaling may improve the survival of hematopoietic progenitors through the expression of anti-apoptotic factors or modulating autophagy or energy metabolism.

Oxidative stress is an important consequence of chronic radiation exposure and affects a myriad of organ systems. The consequences of protracted radiation exposure and oxidative stress are often difficult to study because the detrimental effects of extended radiation exposure shorten the lifespan of cells and animals. We have shown that chloroquine or bone marrow transplantation

can prolong the life span of mice exposed to lethal LDR radiation. Therefore, this model may also be useful in studying the effects of prolonged radiation and oxidative stress on degenerative organ dysfunction or cancer progression in multiple organ systems.⁵¹

**CHAPTER 4: HEDGEHOG SIGNALING ACCELERATES MYELOID DISEASE
PROGRESSION**

Introduction:

Hedgehog signaling cascade²

The Hedgehog (Hh) signaling pathway was first identified in *Drosophila* almost 30 years ago as genetic loci required for proper anterior-posterior segmental patterning.⁵² By examining mutations that disrupt the *Drosophila* larval body plan, Nusslein-Volhard and Weischaus identified several genes that resulted in the duplication of denticles, the spiked cuticular processes located on the anterior half of each body segment, as well as the loss of naked cuticles on the posterior segment. The appearance of a continuous lawn of denticles was reminiscent of the spines of a hedgehog and gave rise to the characteristic name of the soluble factor and signaling pathway responsible for this phenotype. The disruption of antero-posterior positional information within the thoracic and abdominal segments suggested that Hh acts in a wide variety of developmental processes and serves as a classical morphogen to specify cell fates during embryogenesis. Subsequent cloning and study of the *Hh* gene identified it as a unique secreted signaling factor that regulates cell proliferation, migration and differentiation during tissue and organ formation.⁵³ As exemplified by *Drosophila* wing development,⁵⁴ Hh-mediated morphogenesis occurs in a spatial, temporal and dose-dependent fashion. Here, Hh ligand is expressed and secreted by cells within the posterior compartment of the imaginal disc to establish a spatially defined concentration gradient across the anterior segment. Anterior segment cells respond to the local concentration of Hh ligand by modulating the expression of target genes and differentiating into distinct cell types to produce the mature wing.⁵⁵ Hh signaling is widely active during *Drosophila* development, and it is also required for proper formation of the leg and eye.⁵⁶ ⁵⁷ Detailed studies in mice have demonstrated that the Hh pathway is highly conserved as it is required for the development of many organs, especially the skeletal and central nervous

² Reprinted from Crit Rev Eukaryot Gene Expr, Vol 20(2), Lim Y, Matsui W, 129-139, Copyright (2010), with permission from Begell House

systems.⁵⁸ Moreover, in some post-natal organs, the Hh pathway plays an important role in maintaining tissue homeostasis as well as repair and regeneration following injury.⁵⁹

In *Drosophila*, the Hh ligand is initially synthesized as a 45 kDa precursor that undergoes several post-translational modifications to form the active signaling molecule.^{60, 61} A 19 kDa amino-terminal fragment is initially produced by an intramolecular cleavage reaction catalyzed by the carboxy-terminal portion of the precursor. It is then covalently coupled to two lipid molecules, cholesterol and palmitic acid, that further enhance its biologic activity and limit its diffusion within the extracellular space.^{62, 63} These modifications are conserved within all Hh ligands from *Drosophila* to humans.⁶⁴ Cells responding to Hh ligand require two essential proteins, Patched (Ptch), a 12-pass transmembrane protein that serves as the Hh ligand receptor,⁶⁵ and Smoothened (Smo), a 7-pass transmembrane signal transducer.⁶⁶ Unlike many cellular pathways in which receptors function as signal transducers and directly induce pathway activity following ligand binding, Ptch represses Smo and inhibits pathway signaling in the absence of Hh ligand.⁶⁷ Upon ligand binding, the inhibitory effect of Ptch on Smo is relieved and signaling is activated. The nature of the interaction between Ptch and Smo remains poorly understood, but studies have suggested that they do not physically interact within the plasma membrane. Instead, Ptch is thought to catalytically regulate Smo by modulating the production or transport of a small molecule, and recent studies in vertebrates have suggested that oxysterols, including vitamin D3, may serve as this intermediary.⁶⁸⁻⁷⁰ Smo activation ultimately results in activation of Cubitus interruptus (Ci), a zinc finger transcription factor that acts as either an activator or repressor of gene expression depending on its post-translational processing.⁷¹ Cytoplasmic Ci is normally bound to the kinesin-like protein Costal2 (Cos2) allowing interaction with a number of other cellular factors including the serine/threonine protein kinase Fused (Fu), Suppressor of Fused (SuFu), Protein Kinase A, Glycogen Synthase Kinase 3, and Casein Kinase 1.^{72, 73} In the absence of Hh ligand, full-length Ci is phosphorylated and undergoes limited proteolysis that removes the N-terminal transcriptional activation domain.⁷⁴ As a result, truncated Ci enters the nucleus and

acts as a transcriptional repressor. Following the activation of Smo, Ci phosphorylation is altered and full-length Ci induces the expression of Hh target genes. The interpretation of different local concentrations of Hh ligand and specification of Ci transcriptional activity are beginning to be understood. Intermediate levels of Hh ligand result in the binding of Ci to SuFu in the cytoplasm, restricting nuclear import.⁷⁵ Stimulation by high levels of Hh ligand results in the dephosphorylation of Ci, allowing its dissociation from SuFu, and entry into the nucleus.

Divergence in Hedgehog signaling

Despite the functional conservation of the Hh pathway and many of its components, several differences still exist between *Drosophila* and vertebrates. While only one *Hh* gene exists in *Drosophila*, three ligands have been identified in vertebrates, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh).^{53, 76} Dhh is most closely related to *Drosophila* Hh, while Ihh and Shh are more related to one another. Shh is widely expressed in vertebrates including three key signaling centers within the embryo, the notochord, the floor plate, and the zone of polarizing activity.⁷⁷ Hence Shh deficiency is embryonically lethal due to multiple defects in early to mid gestation.⁵³ Ihh is found within hematopoietic cells, bone, cartilage, and the eye, whereas Dhh is primarily expressed in the gonads, external genitalia, eyes and peripheral nerves. The activity of the single Ci transcription factor in *Drosophila* has been expanded to include three homologues, Gli1-3, in mammals.^{78, 79} Gli1 serves as a positive effector of Hh signaling, whereas Gli3 acts as a transcriptional repressor. Gli2 can function as both a positive and negative transcriptional regulator that is specified by both post-transcriptional and post-translational modifications.⁸⁰ The overall output of transcriptional activity is dictated by the balance between activator and repressor forms of these three transcription factors.⁸¹

Cellular localization of Hh pathway components

The cellular localization of pathway components also plays an important role in Hh pathway

activation. In *Drosophila*, Smo is localized within intracellular vesicles in the absence of ligand, then translocated to the plasma membrane upon ligand binding.⁸² In vertebrates, the localization of pathway components occurs within the context of primary cilia.⁸³ In the absence of ligand, Ptch is located within the primary cilia whereas Smo is diffusely found within the plasma membrane.⁸⁴ Following ligand binding, Ptch moves out the primary cilia and Smo moves into the cilia where it interacts with Glis that subsequently enter the nucleus.^{85, 86}

Abnormalities from aberrant Hh signaling

Given the conserved role of the Hh signaling pathway in development, it is not surprising that defects in pathway activity lead to congenital abnormalities in humans.^{58, 87} Holoprosencephaly (HPE) is a cephalic disorder of varying severity characterized by the incomplete cleavage of the forebrain during embryogenesis.⁸⁸ The precise causes of HPE are unknown, but *SHH* haplosufficiency is clearly associated with this disorder in humans. In mice, the loss of a single copy of the *Shh* gene does not lead to HPE, but deletion of both alleles results in cyclopia and fusion of the cerebral hemispheres suggesting that forebrain development in humans is more sensitive to SHH loss than mice.⁸⁹ Mutations in *Ihh* result in brachydactyly that is characterized by shortened phalanges or metacarpals.^{90, 91} *GLI3* mutations have been identified in several congenital malformation syndromes that display characteristic limb abnormalities, including Greig Cephalopolysyndactyly and Pallister-Hall Syndrome.^{92, 93} Mutations that inactivate *PTCH1* are among the best-recognized congenital defects arising from mutations within the Hh signaling pathway. Loss-of-function genetic lesions result in aberrant pathway activity and Gorlin Syndrome characterized by congenital defects of the brain and skeletal system.⁹⁴ Patients with Gorlin Syndrome are also predisposed to develop advanced basal cell carcinomas of the skin, medulloblastomas, and rhabdomyosarcomas suggesting that *Ptch1* functions as a tumor suppressor.⁹⁵⁻⁹⁷ This was the first evidence of aberrant Hh signaling in causing cancers in Gorlin syndrome patients. Interest in the Hh pathway subsequently strengthened by increasing evidence

that the abnormal regulation of the Hh pathway has been widely implicated in tumorigenesis in a variety of organs and tissue systems.^{58, 98} Mutations in regulatory components of the pathway leading to constitutive activation of Hh signaling has also been identified in basal cell carcinoma (BCC) and medulloblastoma (MB).⁹⁹⁻¹⁰³

Hedgehog signaling in hematologic malignancies

In hematologic malignancies, several pre-clinical studies have implicated that Hh signaling may be important in the genesis, relapse and maintenance of these hematologic conditions, and have reinforced interest in using Hh inhibitors in these diseases. In particular, Hh signaling has been shown to play a crucial role in maintaining leukemic stem cells in chronic myeloid leukemia (CML), and the effects of *Smo* deletion in CML have been explored using mouse models. Zhao *et al* used the Vav-Cre-Lox system to isolate *Smo* deficient HSCs from *Smo* deficient mice, whereas Dierks *et al* used fetal liver cells from *Smo* deficient mouse embryos.^{104, 105} Both groups virally expressed *BCR-ABL* in these cells before transplanting them into recipient mice. They found that *Smo* deletion reduced leukemic stem cell numbers and decrease the incidence of leukemia. Pharmacologic inhibition also decreased leukemic growth in diseased mice. They showed that Hh signaling is critical to maintaining the leukemic stem cell population in CML, and can be therapeutically targeted in the treatment of CML. Similarly, in multiple myeloma, the disease initiating cells express constituents of the Hh pathway, and *Smo* inhibitors had the ability to reduce the population of these cells.¹⁰⁶ Additionally, in precursor B-cell acute lymphoid leukemia (B-ALL), *Smo* inhibition also reduced self-renewal both *in vitro* and *in vivo*.¹⁰⁷ Although Hh signaling plays a role in maintaining disease initiating cell self-renewal in these diseases, less is known about its role in acute myeloid leukemia (AML). Studies have shown that Mx1Cre deleted *Smo* is dispensable in a *MLL-AF9* induced AML mouse model as well as Notch dependent T-cell acute lymphoid leukemia (T-ALL) model, suggesting that targeting Hh signaling may not be sufficient in these acute leukemias.^{108, 109} In the AML model, there was no

difference in phenotype as assayed by *in vitro* serial plating assays, *in vivo* AML disease penetrance or latency compared to the control *Smo*^{+/+} cells, in contrast to the findings in CML models. In the T-ALL model, there was no change in the kinetics of disease onset or secondary transplants by genetic deletion or pharmacological inhibition of Smo. Nevertheless, components of the Hh signaling pathway have been shown to be expressed in leukemia cell lines and patient samples. Recently, a study using a Smo inhibitor manufactured by Pfizer showed that antagonizing Hh signaling reduces dormant leukemic stem cell activity in AML patients, suggesting that Hh inhibition may have some efficacy in leukemia. Hh inhibition also enhanced tyrosine kinase inhibitor sensitivity, providing a strong rationale for combinatorial therapy in leukemia. These studies suggest a role of Hh signaling in being part of a core machinery driving myeloid leukemic stem cell maintenance in both CML and AML.

FLT3-ITD mutations in AML

FMS-like tyrosine kinase 3 (FLT3) is a class III receptor tyrosine kinase that is expressed on early hematopoietic stem/progenitor cells, and is vital for the development of normal levels of mature myeloid and lymphoid cells.¹¹⁰ On binding with the FLT3 ligand (FL), FLT3 is activated, which involves receptor dimerization and kinase activity by activating multiple downstream signaling pathways, including Ras/MAP kinase, AKT and STAT5.^{111, 112} High coexpression of FLT3 with FL may lead to dysregulation of activity via autocrine or paracrine mechanisms that promote leukemogenesis, as observed in MLL-rearranged infant ALL.^{113, 114} FLT3 mutations represent one of the most common molecular perturbations in acute myeloid leukemia (AML), accounting for 30–35% of *de novo* cases.^{115, 116} Mutations of FLT3 lead to constitutive kinase activation. These mutations generally occur in the juxtamembrane (JM) domain or in the tyrosine kinase domain (TKD). The JM mutations factor in approximately 23% of newly diagnosed cases of AML and occur as in-frame internal tandem duplications (ITDs) of varying length, resulting in duplication of a sequence ranging typically from 4–50 amino acids, often accompanied by a one or two

amino-acid insert. The JM domain functions as an autoinhibitory mechanism to regulate FLT3 kinase activity, and disruption by mutations destabilize its conformation, leading to constitutive signaling.¹¹⁷ Point mutations to the kinase domain occur lower in frequency. A recent study also reported that these D835Y point mutations result in less aggressive disease compared to the ITD mutations in a knock-in mouse model.¹¹⁸ The presence of FLT3-ITD mutations confers a poor prognosis to patients with AML.^{119, 120} Hence there is great interest in developing FLT3 inhibitors as a form of treatment for AML, and also in better understanding disease transformation in FLT3-ITD driven leukemia.

Our hypothesis

Incidentally, activating or inhibiting mutations in components of the Hh pathway have not been found in hematopoietic malignancies; current data suggests that in most hematopoietic neoplasms, Hh signaling does not play an independent role in tumor initiation, but instead contributes to tumor maintenance, growth, drug resistance and cancer stem cell survival. Perhaps Hh inhibition may work better in advance or relapsed AML, or in AML driven by certain mutations, such as FLT3 mutations. More studies in the mechanistic role of Hh signaling in AML will be crucial in improving the use of Hh inhibitors in this devastating disease. We aim to study the role of Hh signaling in AML pathogenesis, and show evidence of an efficient use of Smo inhibition in combinatorial therapy. We found evidence of increased Hh signaling in secondary human AML patient peripheral blast samples, as well as advanced CML compared to the initial stages of disease. Thus, we hypothesize that the Hh pathway plays a role in accelerating disease progression. To model chronic myeloid proliferation, we used the FLT3-ITD; Mx1Cre mouse, where fatal myeloproliferative disease, initiated by an activating mutation of the FLT3 tyrosine kinase receptor, develops with a median time of 10 months.¹²¹ We bred these FLT3-ITD mice with mice harboring the *SmoM2* allele, and conditionally activated both alleles in the hematopoietic system in adult mice by way of Mx1Cre to interrogate the role of Hh

signaling in accelerating leukemia progression. We found that SmoM2; FLT3-ITD; Mx1Cre mice (referred to as SmoM2; FLT3-ITD) developed rapidly fatal acute myeloid leukemia with 100% penetrance by 3 months. The disease is characterized by elevated white blood cell counts, splenomegaly, increased Gr1⁺ cKit⁺ cells in the bone marrow, and Mac1⁺ Gr1⁺ cells in the peripheral blood. We also found that the increased Hh signaling in these mice enhanced the proliferation of myeloid progenitors, thus contributing to disease progression. Inhibiting both the Hh and FLT3 signaling pathways with specific inhibitors in FLT3-ITD AML cell lines decreased cell growth and clonogenicity, and caused cell cycle progression arrest. Furthermore, combined treatment using both the Hh and FLT3 inhibitors *in vivo* significantly improved overall survival of diseased mice. This model sheds light on the role of developmental signaling pathways in accelerating cancer progression, and also provides rationale for combinatorial therapy with both FLT3 and Hh pathway inhibitors.

Method:Patient samples

Human AML and normal bone marrow and peripheral blood samples were collected under an institutionally approved protocol with informed patient consent in accordance with the Declaration of Helsinki. Samples were obtained prior to therapy and/or at relapse. Mononuclear cells were isolated by Ficoll centrifugation and cryopreserved in liquid nitrogen until use. Total RNA was extracted from mononuclear cells using RNeasy Plus kit (Qiagen), and used for cDNA synthesis for qRT-PCR analysis.

Mice

FLT3-ITD; Mx1Cre mice harboring a FLT3-ITD mutation knocked into the endogenous FLT3 locus were previously described.¹²¹ These mice were bred with SmoM2 mice purchased from the Jackson Laboratories to generate mice with the FLT3-ITD, SmoM2 and Mx1Cre alleles. Female congenic C57BL/6 CD45.1 recipient mice, 4 – 6 weeks old, were purchased from the National Cancer Institute (NCI). All mice were housed in a pathogen-free animal facility at the Johns Hopkins University School of Medicine. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Generation and analysis of diseased mice

DNA was extracted from tail fragments obtained from mice upon weaning. The genotypes of the transgenic mice were determined by performing PCR reactions on the extracted DNA using the REDExtract kit (Sigma) according to manufacturer's protocol with the following primers.

<u>Genotyping primers</u>	Forward	Reverse
SmoM2	5'- AAG TTC ATC TGC ACC ACCG -3'	5'- TCC TTG AAG AAG ATG GTG CG -3'
FLT3-ITD	5'- CTC TCG GGA ACT CCC ACT TA -3'	5'- TGC AGA TGA TCC AGG TGA CT -3'
Mx1Cre	5'- GCG GTC TGG CAG TAA AAA CTA TC -3'	5'- GTG AAA CAG CAT TGC TGT CAC TT -3'

Mice harboring alleles heterozygous for both *SmoM2* and FLT3-ITD were obtained. All mice used in the study also had the *Mx1Cre* allele. Littermates with just one copy of the *SmoM2* or FLT3-ITD alleles were used as controls. Wild type controls had the *Mx1Cre* allele only. Upon weaning and genotyping, the mice were then administered with 5 doses of 300ug poly(I:C) via intraperitoneal injections every other day. 4 weeks after the last poly(I:C) injection, peripheral blood was obtained by retro-orbital bleeding using heparinized microcapillary tubes (Fisher). Complete blood count analysis was obtained using the Hemavet 950 Hematology Analyzer (Drew Scientific). Peripheral blood was then lysed with red blood cell (RBC) lysis buffer, and DNA was extracted. Successful excision of the transgenes was confirmed by performing PCR reactions on peripheral blood DNA with primers for the loxp sites.

	Forward	Reverse
loxp	5'- CTT CGT ATA ATG TAT GCT ATA CG -3'	5'- TCG TAT AGC ATA CAT TAT ACG -3'

Peripheral blood mononucleocytes were also resuspended in PBS and analyzed using the FACS Calibur for expression of YFP. Development of disease was assessed by the presence of Mac1⁺ Gr1⁺ cells in the peripheral blood and Gr1⁺ cKit⁺ cells in the bone marrow by flow cytometry. Mice were monitored for lethargy, hunching, weight loss, and sacrificed and analyzed upon moribund. Bone marrow was flushed from the femurs and tibias, passed through a 70uM filter to obtain single cell suspensions, and red blood cells were lysed with ammonium chloride buffer. The bone marrow cells were then stained with a variety of cell surface markers for flow cytometry analysis. Hematoxylin and eosin staining of formalin-fixed tissues and organs such as the bone marrow and spleen was performed. Blood smears were also obtained and stained with Wright-Giemsa stain.

Antibodies and FACS analysis

Antibodies used for peripheral blood analysis were obtained from eBioscience and include the following: Mac1, Gr1, B220 and CD3. Bone marrow lineage cocktail includes the following biotinylated antibodies: Gr1, Ter119, B220, CD3. Bone marrow cells were also stained with strepavidin-V500, cKit-PE-CF594, Sca1-APC-Cy7, CD16/32-AF700, CD34-PacBlue, FLT3-APC, CD150-Qdot405 (BioLegend), CD48-PE, and IL7R-PerCP-Cy5.5 for analysis of hematopoietic stem and progenitor cell populations. FACS analysis was performed on the FACS Caliber and the LSR II (BD Biosciences) and analyzed using FlowJo software (FlowJo).

Bone marrow transplantation

Bone marrow from unexcised SmoM2; FLT3-ITD; Mx1Cre mice were obtained by flushing the tibia and femur bones with PBS, and then passing the bone marrow cells through a 70uM filter. 5×10^6 whole bone marrow cells were transplanted into lethally irradiated recipients through retroorbital injections. These recipients are congenic C57BL/6 CD45.1⁺ female mice of 4 – 6 weeks of age, treated with a split dose of 1200 rads of lethal total body irradiation – 800 rads and

400 rads about 3 hours apart, prior to transplantation. 4 weeks following bone marrow transplantation, chimerism of the mice were determined by staining peripheral blood with CD45.1 and CD45.2 antibodies, and analyzing percentage of CD45.2 by flow cytometry. Following confirmation that all mice should have at least 50% chimerism, poly(I:C) was then administered in 5 doses every other day, similar to transgenic animals as previously described, to excise the transgenes in the engrafted CD45.2 population.

In vivo BrdU analysis

In vivo BrdU incorporation experiments were conducted using the FITC BrdU Flow kit (BD Biosciences). SmoM2; FLT3ITD; Mx1Cre animals were injected intraperitoneally with 150ug of BrdU solution about 3 months after they were induced with poly(I:C). The mice were sacrificed 10 hours after administration of BrdU, and bone marrow was collected from the femurs. Cell surface staining was performed on whole bone marrow cells obtained from one femur prior to fixation, permeabilization, DNase treatment and then staining was performed with anti-BrdU antibody according to instructions provided in the manufacturer's protocol. At least 3 independent experiments were conducted using littermate controls.

Quantitative Real-time PCR analysis

Total RNA was extracted using the RNeasy Plus Mini kit (Qiagen), and used for cDNA synthesis using Superscript III reverse transcriptase (Invitrogen). qRT-PCR was carried out using SYBR Green and Taqman mastermixes using the following primers (ABI). Gene expression levels were normalized against mouse *Rps16* and human Beta-Actin expression and compared to wild type control using the $\Delta\Delta CT$ method of analysis.

SYBR Green qRT-PCR primer sequences:

	Forward	Reverse
Mouse <i>Flt3</i>	5'- GCT CTG TCT CCC CTT CAT TG -3'	5'- CCA GGA CCT TCC CAA ACT CT -3'
Mouse <i>Rps16</i>	5'- CTT GGA GGC TTC ATC CAC AT -3'	5'- ATA TTC GGG TCC GTG TGA AG -3'
Human <i>GLII</i>	5'- GGC TGC AGT AAA GCC TTC AGC AAT -3'	5'- TGC AGC CAG GGA GCT TAC ATA CAT -3'
Human Beta-Actin	5'- ATC CAG GAA ACT ACC TTC AAC TCC AT -3'	5'- CAT ACT CCT GCT TGC TGA TCC ACA TC -3'

Taqman qRT-PCR primers (from ABI):

hPTCHI: Hs00181117_m1

hGLII: Hs01110766_m1

FACS sorting of KSL and GMP cells

For FACS sorting of KSL and GMP cells, whole bone marrow was first collected by obtaining hind limbs, hips and spine bones and crushing them in 2%FBS/PBS using a mortar and pestle. Lineage-negative cells were isolated using a mixture of biotin-labeled monoclonal antibodies against CD3e, B330, Gr1 and TER-119. The cells were then incubated with anti-biotin microbeads (Miltenyi Biotec) for 20 mins at 4°C, and washed with PBS. The cell pellet was resuspended in 500ul of Miltenyi buffer, and lineage positive cells were depleted by passing the cell suspension through a LD column attached to a Miltenyi AutoMACS magnetic separator (Miltenyi Biotec). Lineage negative cells were then immunostained with streptavidin, cKit, Sca1, IL7R, CD34, CD16/32 antibodies (eBioscience). KSL (cKit⁺ Sca1⁺ Lin⁻) and GMP (cKit⁺

Sca1- Lin- IL7R- CD34+ CD16/32+) cell populations were sorted on a BD FACSAria cell sorter (BD Biosciences).

Microarray analysis

KSL and GMP cells were FACS sorted (BD Aria) from wildtype, FLT3-ITD and SmoM2; FLT3-ITD animals at approximately 3 months of age, or when SmoM2; FLT3-ITD animals were moribund. Total RNA from these cell populations were extracted using the RNeasy Plus Micro kit (Qiagen), and hybridized onto the Affymetrix Mouse genome 430 2.0 array (Affymetrix). Gene set enrichment analysis (GSEA) was then performed.¹²²

Statistics

Log-rank (Mantel-Cox) test p-values for the Kaplan-Meier survival curves were generated using GraphPad Prism. All other p values were calculated using an unpaired two-tail Student's t test, unless otherwise specified. All data were collected from at least 3 independent experiments, and presented as the mean \pm standard deviation.

Results:

Hedgehog signaling pathway is clinically relevant in human AML

To validate the presence of Hh signaling in human myeloid disease progression, we obtained patient samples at various stages of disease and examined Hh target gene expression by qRTPCR. In patients with CML, there were consistently higher levels of *GLII* and *PATCH1* gene expression upon disease progression from chronic phase to blast crisis compared to normal hematopoietic stem cells (Figure 4.1A). In addition, 4 out of 6 patients who progressed from myelodysplastic syndromes (MDS) to secondary AML had 2 to 9 fold higher levels of *GLII* and *PATCH1* upon developing AML (Figure 4.1B). Intriguingly, patients with FLT3-ITD AML also had significantly higher levels of *GLII* expression compared to normal human CD34+ bone marrow cells (Figure 4.1C), suggesting the relevance of Hh signaling in FLT3-ITD AML. Although not much is currently known about the precise role of Hedgehog signaling in human acute myeloid leukemia, we have shown evidence for the clinical relevance of Hedgehog signaling in human myeloid leukemia progression and advanced human FLT3-ITD AML, and speculate that Hh signaling may play a role in accelerating disease progression. To further mechanistically investigate our hypothesis that Hedgehog signaling plays a role in myeloid disease progression and transformation, we generated a novel mouse model of myeloid disease progression by conditional activation of both Hh signaling and FLT3-ITD.

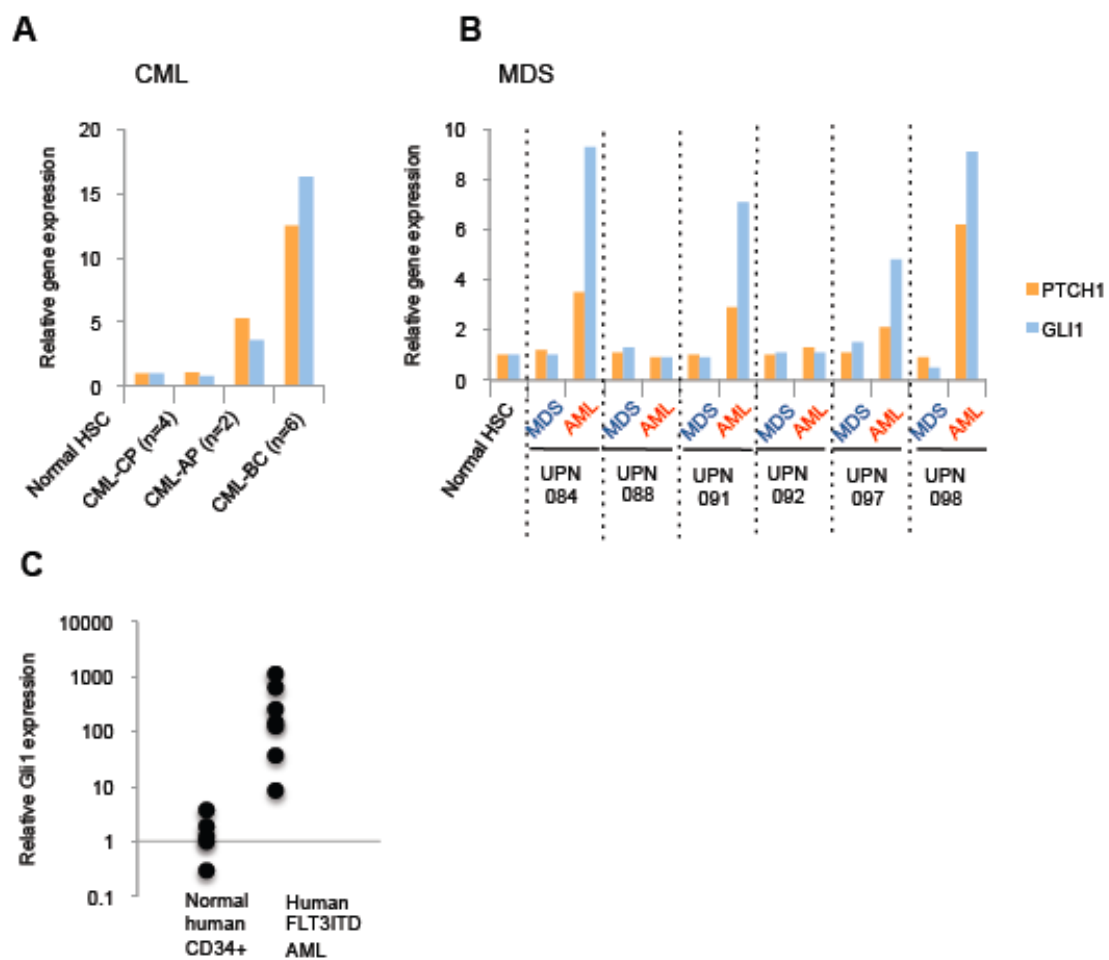
Figure 4.1: Hedgehog signaling increases with disease progression and transformation in human myeloid malignancies

(A)Relative Hedgehog pathway target gene expression in CML patients

(B)Relative Hedgehog pathway target gene expression in 6 secondary AML patients

(C)Relative *GLII* expression by qRTPCR in human FLT3-ITD AML samples compared to normal CD34+ bone marrow

Figure 4.1



SmoM2; FLT3-ITD; Mx1Cre mice die rapidly of acute myeloid leukemia

Initially identified in human basal cell carcinoma, the activating mutation W535L in human Smoothed (SMOM2) causes constitutive, ligand-independent activation of the Hedgehog (Hh) pathway.⁹⁹ To investigate whether Hh signaling causes disease progression, we used a previously described mouse model *R26-SmoM2* in which a cDNA fragment encoding the SmoM2-YFP fusion protein was targeted into the ubiquitously expressed *Rosa26* locus (*R26*) behind a stop cassette flanked by Loxp sites.¹²³ The yellow fluorescent protein (YFP) fused at the C terminus of SmoM2 has been shown to not interfere with its activity in neural epithelium and provides a tool to trace the lineage of all Cre-expressing cells and their progeny.¹²⁴ To activate Hh signaling during definitive hematopoiesis, we crossed the *R26-SmoM2* mice with Mx1Cre transgenic mice.¹²⁵ These mice have normal hematopoiesis. To investigate the role of Hh signaling in myeloid disease progression, we crossed the SmoM2; Mx1Cre mice with a previously established mouse model of chronic fatal myeloproliferation by a knock-in of FLT3-ITD.¹²¹ FLT3-ITD; Mx1Cre mice develop fatal myeloproliferative disease with a median survival of 10 months, but never transform to acute leukemia. The FLT3-ITD knock-in mice were generated by the insertion of an 18 base pair human internal tandem duplication (ITD) mutation into the juxtamembrane domain of murine FLT3 behind an inverted neo cassette flanked by loxp sites. SmoM2; FLT3-ITD; Mx1Cre mice generated from the cross were treated with poly(I:C) upon weaning to induce the transgenes post-natally in the hematopoietic system.

Genotyping was confirmed by PCR (Supplementary Fig 1), and successful excision of the FLT3-ITD and *SmoM2* alleles were determined by PCR and flow cytometry analysis of peripheral blood (Supplementary Fig 2). Intriguingly, while Hedgehog signaling has been found to have no impact on normal adult hematopoiesis, we found that inducing constitutively active Hedgehog signaling via SmoM2 together with FLT3-ITD causes rapidly fatal acute myeloid leukemia with 100% penetrance.^{108, 109} SmoM2; FLT3-ITD animals died in 3 months compared to FLT3-ITD animals

(Figure 4.2A). The disease was characterized by increased bone marrow cellularity (Figure 4.2B), spleen weights and sizes (Figure 4.2C), elevated white blood cell counts (Figure 4.2D) in moribund *SmoM2*; *FLT3-ITD* animals compared to age-matched controls. There was also an increased population of $\text{Mac1}^+ \text{Gr1}^+$ myeloid cells in the peripheral blood (Figure 4.2E) and accumulation of $\text{Gr1}^{\text{mid}} \text{cKit}^+$ cells in the bone marrow (Figure 4.2F). Hematoxylin and eosin staining of the spleen and bone marrow showed complete effacement of splenic architecture and accumulation of immature myeloid cells in both organs. Bloods smears showed accumulation of immature leukemic blasts, and are suggestive of acute myeloid leukemia (Figure 4.2G). Furthermore, there was infiltration of similar immature myeloid cells into non-hematopoietic organs such as the liver and lung in the moribund *SmoM2*; *FLT3-ITD* animals (Supplementary Figure 3). According to the Bethesda proposals for classification of nonlymphoid hematopoietic neoplasms in mice as determined by the hematopathology subcommittee of the Mouse Models of Human Cancers Consortium, the *SmoM2*; *FLT3-ITD* mice have an MPD-like myeloid leukemia, as characterized by rapid death in the primary animal, at least 20% immature myeloid blasts in the peripheral blood, infiltration of myeloid cells into non-hematopoietic organs, and mature myeloid cells in the peripheral blood.¹²⁶

To verify that Hedgehog signaling is active in the animals, we looked at the levels of gene expression of *Gli1*, a target of the Hedgehog signaling pathway, by qRT-PCR, in bone marrow cells. Animals harboring the *SmoM2* allele show significantly elevated *Gli1* expression in the bone marrow, suggesting that Hedgehog signaling is driving the disease phenotype (Figure 4.2H). Loss of heterozygosity of the wild-type *FLT3* allele has been reported to contribute to transformation and progression of disease.¹²⁷ We observed by PCR analysis of bone marrow samples that the *SmoM2*; *FLT3-ITD* mice did not have the loss of heterozygosity despite disease transformation, implying that Hedgehog signaling may be facilitating a different mechanism to *FLT3* mediated disease progression (Supplementary Fig 4).

Figure 4.2. SmoM2; FLT3-ITD; Mx1Cre animals die rapidly of acute myeloid leukemia

(A) Kaplan Meier survival curve of transgenic animals (log-rank $P < 0.001$)

(B) Bone marrow cellularity of animals at around 3 months after PIPC (n=3 to 7 for each genotype) * $p < 0.05$, ** $p < 0.01$

(C) Spleen weights and representative spleens at 3 months ** $p < 0.01$

(D) White blood cell counts at 3 months *** $p < 0.001$

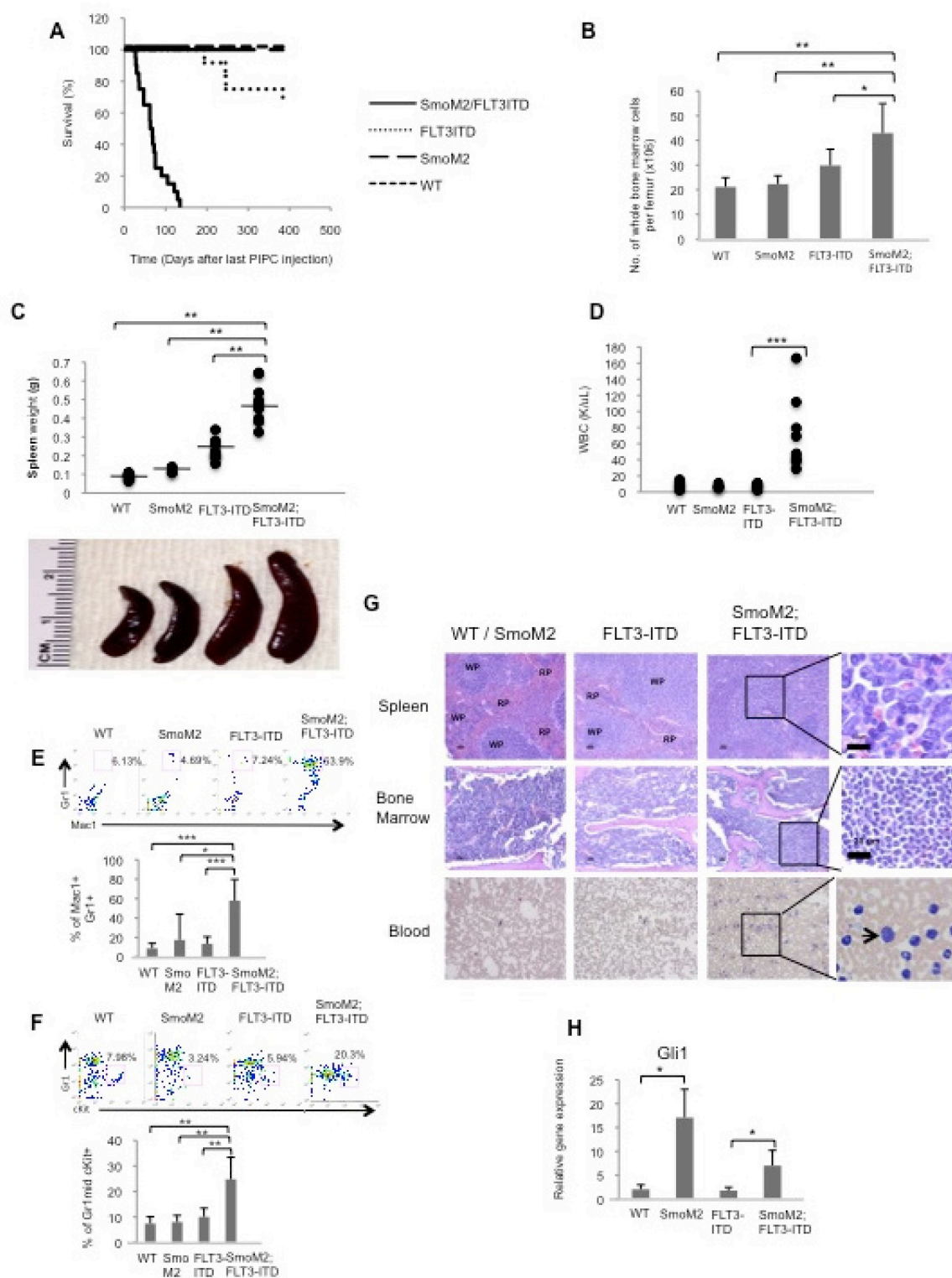
(E) FACS analysis of disease phenotype in peripheral blood. Bar graphs depict percentage of Mac1⁺ Gr1⁺ cells in peripheral blood. * $p < 0.05$ *** $p < 0.001$

(F) FACS analysis of disease phenotype in bone marrow. Bar graphs depict percentage of Gr1^{mid} cKit⁺ cells in total bone marrow. ** $p < 0.01$

(G) Hematoxylin and eosin (H&E) staining of spleen, bone marrow. Wright-Giemsa staining of peripheral blood smear.

(H) qRT-PCR analysis showing relative Gli1 gene expression in whole bone marrow * $p < 0.05$

Figure 4.2



Myeloid progenitor compartments are expanded in diseased SmoM2; FLT3-ITD; Mx1Cre mice

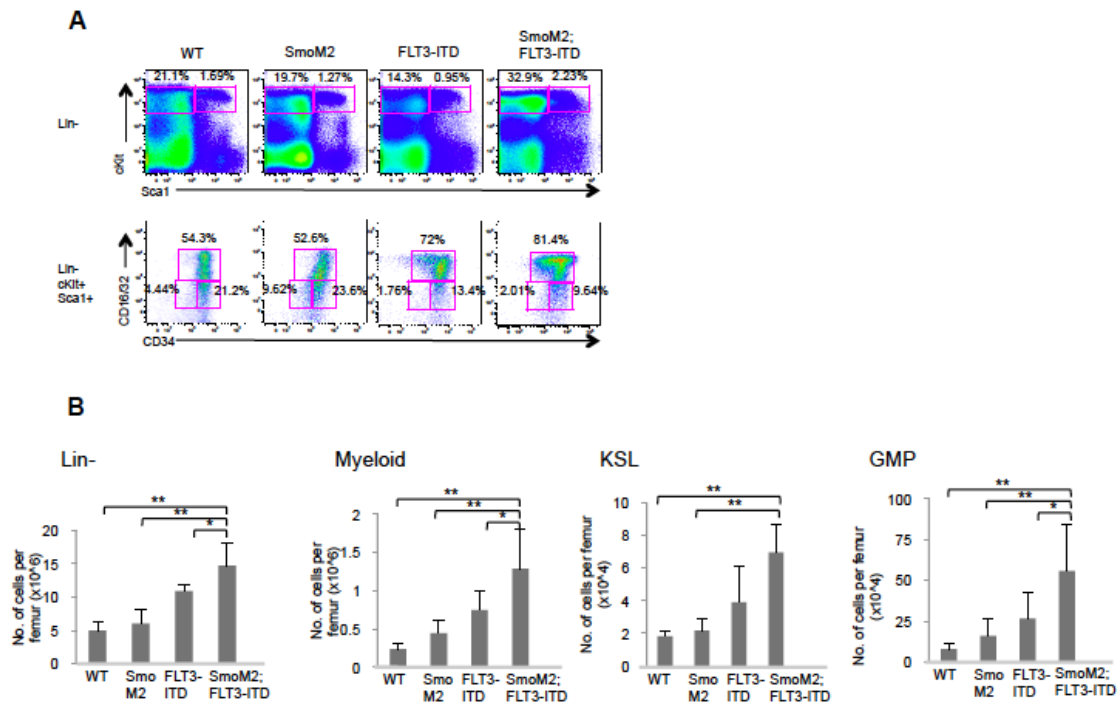
To characterize the disease phenotype on a cellular level, we examined the hematopoietic stem and progenitor subsets in the bone marrow by FACS analysis. Bone marrow was harvested from the femur and stained with cell surface markers. On examination of hematopoietic stem and progenitor cell populations, the SmoM2; FLT3-ITD mice consistently showed an increase in lineage negative progenitors. Although there was no significant difference in the number of KSL cells – a population enriched for multipotent hematopoietic stem cells, the myeloid progenitors and GMPs were significantly expanded by 2-fold in the SmoM2; FLT3-ITD mice compare to FLT3-ITD mice (Figure 4.3A, B). On further examination of more primitive hematopoietic stem cell populations within the KSL compartment by FLT3 and CD34 expression, there were no significant differences in the frequencies of long-term HSC (LT-HSC), short-term HSC (ST-HSC), and MPP cells. The CMP compartment was also not significantly expanded (Supplementary Fig 5). A recent report showed that the FLT3-ITD myeloproliferative neoplasm was caused by increased proliferation in the normally quiescent primitive LT-HSC and SLAM (Lin- CD150+ CD48-) cells, leading to depletion of these primitive hematopoietic stem cells.¹²⁸ Compared to FLT3-ITD mice, the SmoM2; FLT3-ITD mice have a smaller population of Lin- CD150+ CD48- cells. This suggests that Hedgehog signaling may contribute to disease progression by expanding the myeloid progenitors at the expense of primitive hematopoietic stem cells.

Figure 4.3. Myeloid progenitor compartments are expanded in diseased SmoM2; FLT3-ITD; Mx1Cre animals

(A) Representative FACS plots depicting relative percentage of stem and progenitor (Lin⁻ Sca1⁺ cKit⁺), myeloid progenitor (Lin⁻ Sca1⁻ cKit⁺), GMP (Lin⁻ Sca1⁻ cKit⁺ CD34⁺ CD16/32⁺), CMP (Lin⁻ Sca1⁻ cKit⁺ CD34⁺ CD16/32⁻) and MEP (Lin⁻ Sca1⁻ cKit⁺ CD34⁻ CD16/32⁻) cells in bone marrow of transgenic animals.

(B) Bar graphs show number of cells from each population per femur from at least 3 animals per genotype. *p<0.05, **p<0.01

Figure 4.3



SmoM2 and FLT3-ITD cooperate to cause accelerated disease in a cell autonomous manner

To determine if the observed disease phenotype in the SmoM2; FLT3-ITD mice was due to the direct expression of both transgenes in the hematopoietic cells in the bone marrow or if it was influenced by the microenvironment, we transplanted unexcised CD45.2 SmoM2; FLT3-ITD; Mx1Cre bone marrow into lethally irradiated wild-type CD45.1 recipients and administered the chimeric mice with poly(I:C) 3 weeks after transplant (Supplementary Fig 6). Successful excision of the transgenes was verified in the chimeric animals by PCR and flow cytometry of peripheral blood. These mice developed disease only in the CD45.2 SmoM2; FLT3-ITD population, and not in the normal host CD45.1 population. Compared to transgenic SmoM2; FLT3-ITD mice characterized in the previous figure, we observed that these chimeric mice died in a similar time frame, with the same blood and bone marrow phenotype in the CD45.2 population, suggesting that the disease occurs in a cell autonomous manner in hematopoietic cells.

Myeloid progenitors proliferate more in mice with both FLT3-ITD and SmoM2 compared to FLT3-ITD alone

To mechanistically explain the increased cellularity and myeloid progenitor expansion resulting in disease acceleration and rapid death in mice with both *SmoM2* and FLT3-ITD alleles, we decided to look at the proliferative capacity of these myeloid progenitors by BrdU incorporation *in vivo*. Transgenic animals at 3 months post poly(I:C) induction were injected with BrdU and bone marrow was harvested 10 hours later and co-stained with cell surface markers and anti-BrdU antibody to determine proliferation in different myeloid progenitor compartments. While there were no significant differences in BrdU incorporation in the more primitive hematopoietic stem cell compartments (Supplementary Fig 7), SmoM2; FLT3-ITD myeloid progenitor cells had a higher level of BrdU incorporation compared to mice having just FLT3-ITD alone (Figure 4.4A, B).

We also performed a gene expression profiling by microarray of FACS-sorted KSL and GMP cells from 3 month old wild-type, FLT3-ITD and SmoM2; FLT3-ITD mice. Gene Set Enrichment Analysis (GSEA) of the microarray data indicated that there was significantly enhanced proliferation as well as Stat5 signaling in SmoM2; FLT3-ITD mice compared to FLT3-ITD alone (Figure 4.4C).¹²² The Stat5 signaling pathway is the main downstream target of FLT3-ITD, and has been shown to play a role in maintaining the survival and proliferation of myeloid leukemia cells. Compared to wild-type mice, FLT3-ITD mice have increased Stat5 signaling, as expected. Our data suggests that Hedgehog signaling may enhance the proliferation and expansion of myeloid progenitors by amplifying FLT3-ITD mediated Stat5 signaling. Specifically, *JunB* and Krüppel-like factor 6 (*Klf6*) genes were found to be consistently upregulated in the SmoM2; FLT3-ITD samples. Although these genes function as tumor suppressors in certain cancers, these effects are highly dependent on cell type and tissue. Interestingly, cooperation between *GLI* and *JUN* family transcription factors in human keratinocytes suggests that the *GLI-JUN* interaction may play a significant role in tumor progression.¹²⁹ In addition, *KLF6* has been reported to play a role in developmental hematopoiesis, and similar to Hh, it may also cooperatively regulate the oncogenic EGFR pathway.^{130, 131} We validated these genes by performing qRTPCR on sorted GMPs (data not shown). Interestingly, we also observed that *Flt3* gene expression levels were significantly higher in the GMPs of SmoM2; FLT3-ITD mice compared to FLT3-ITD only mice (Figure 4.4D). This suggests that Hh signaling can augment the already constitutively active FLT3 signaling to accelerate disease progression. This in turn enhances downstream Stat5 signaling. While Hedgehog signaling has no impact on normal hematopoiesis, it appears to contribute to disease progression in the context of existing oncogenes. In this case, Hedgehog signaling cooperates with and amplifies FLT3-ITD mediated signaling pathways to cause myeloid disease acceleration by increasing proliferation of myeloid progenitors.

Figure 4.4. Myeloid progenitors proliferate more in mice with both FLT3-ITD and SmoM2 compared to FLT3-ITD alone

(A) Representative FACS plots depicting relative percentage of cells with BrdU incorporation in GMP (Lin⁻ Sca1⁻ cKit⁺ CD34⁺ CD16/32⁺), CMP (Lin⁻ Sca1⁻ cKit⁺ CD34⁺ CD16/32⁻) and MEP (Lin⁻ Sca1⁻ cKit⁺ CD34⁻ CD16/32⁻) compartments

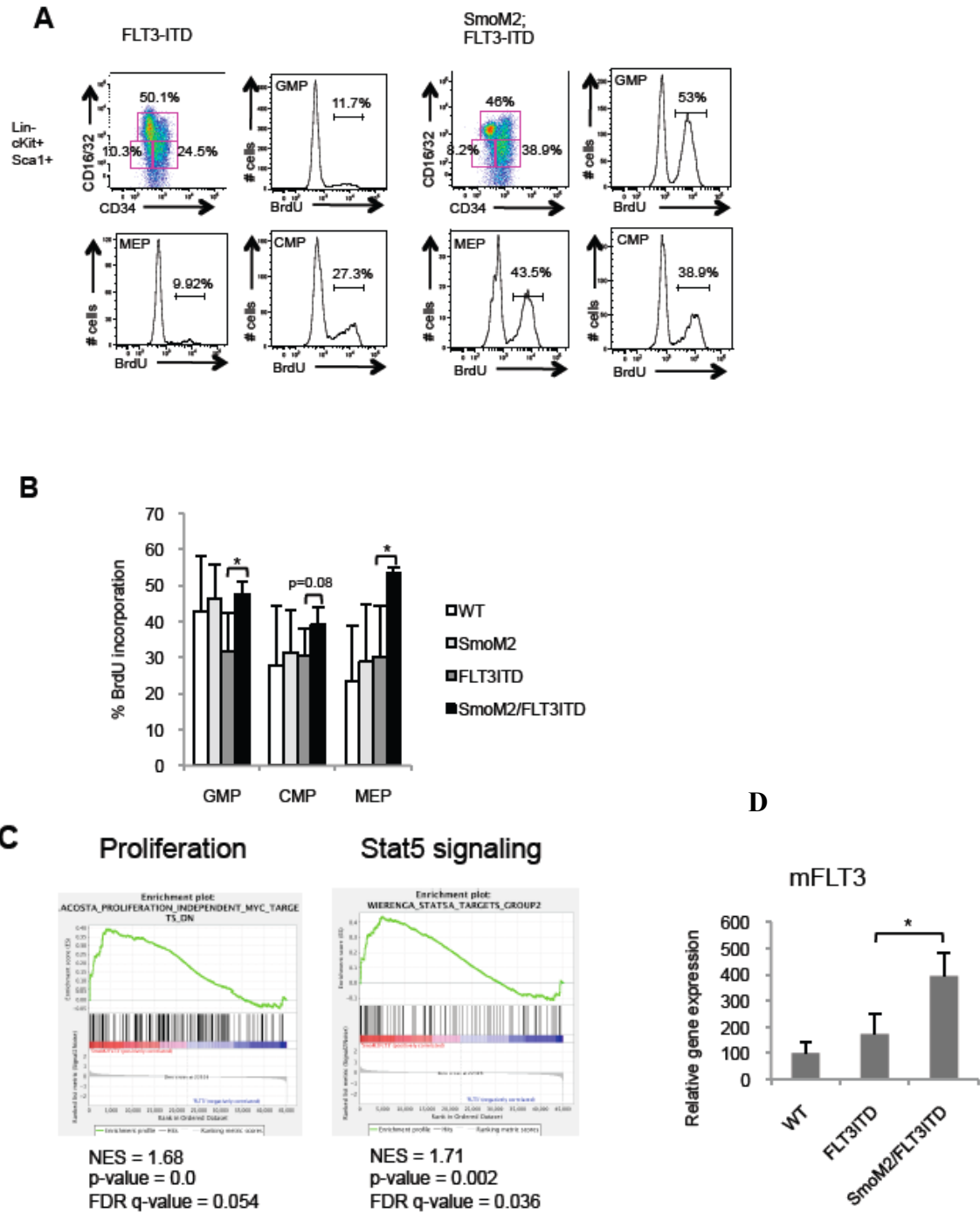
(B) Bar graphs show percentage of cells with BrdU incorporation from each population *p<0.05, n=3 for each genotype

(C) GSEA analysis of SmoM2;FLT3-ITD compared to FLT3-ITD bone marrow cells

(D) qRT-PCR of relative mouse FLT3 gene expression levels in sorted mouse GMP cells.

*P<0.05, n=3 for each genotype

Figure 4.4



Discussion:

Myeloproliferative disease progression

Myeloproliferative neoplasms are hematologic disorders characterized by terminal myeloid cell expansion in the peripheral blood, with a long-term risk of transformation to acute leukemia. Generally the incidence of myeloproliferative diseases increases with age, and patients inevitably develop secondary myeloid malignancies such as acute myeloid leukemia (AML). Conventional high dose chemotherapy is both a toxic and ineffective form of therapy. Bone marrow transplantation may present a cure, however most patients are at an advanced age or have coexisting diseases and are therefore poor transplant candidates. Aberrant signal transduction appears to be responsible for disease pathogenesis in proliferative myelodysplastic syndrome (MDS) subtypes. Most of what is currently known about myeloid disease progression came from studies on and treatment of chronic myeloid leukemia (CML). CML is a clonal myeloproliferative disease characterized by an accumulation of myeloid cells. In the initial chronic phase (CP), there is accumulation of myeloid progenitors and mature myeloid cells in the blood and extramedullary tissues.¹³² Most patients are diagnosed at this stage; CML-CP is also characterized by the presence of the *BCR-ABL1* fusion oncogene, also known as the Philadelphia chromosome. The advance phase of CML includes an initial accelerated phase (AP), where patients may still respond to treatment, and a subsequent more aggressive blastic phase (BP) characterized by a maturation arrest with a median survival of 6 months. Molecular mechanisms underlying disease progression from CP to BP are currently poorly understood. Although the loss of p53 and reduplication of the Philadelphia chromosome are the most frequent abnormalities leading to the development of blast crisis, there are no well-characterized mechanisms and events that overwhelmingly contribute to disease progression. Activating mutations in the receptor tyrosine kinase FLT3 is rare in MDS, but is associated with leukemia progression and frequently found in AML. To study myeloid disease progression, we used the previously published FLT3-

ITD; Mx1Cre mouse model, where animals developed fatal myeloproliferative disease over a prolonged time period, and bred it to a SmoM2 mouse, where Smo is driving constitutive Hh signaling.

We generated a novel mouse model of myeloid disease progression by conditionally activating SmoM2 and FLT3-ITD in the hematopoietic system, and showed that increased Hedgehog signaling accelerated myeloid disease progression initiated by FLT3-ITD. SmoM2; FLT3-ITD mice developed a rapidly fatal myeloid leukemia characterized by increased proliferation of myeloid progenitors. Intriguingly, SmoM2 alone does not impact normal hematopoiesis, hence we provide evidence that a developmental signaling pathway such as the Hh pathway can function as a disease accelerator in the presence of an existing oncogene, in this case, FLT3-ITD. While disease acceleration and transformation in FLT3-ITD leukemias are often caused by the loss of the wild-type *FLT3* allele, we show that the Hedgehog signaling pathway can also facilitate disease progression without the loss of heterozygosity and genetic instability.^{127, 133} Disease progression also occurs in a cell autonomous manner in our model.

Mechanisms of FLT3-ITD AML transformation

Although specific lesions and mutations are known to cause AML, the mechanisms of disease pathogenesis are still unclear. Evidence from retroviral bone marrow transplantation and transgenic mouse models have shown that additional oncogenes and mutations are required to cooperate with FLT3-ITD mutations to cause leukemia. Particularly, FLT3-ITD mutations together with chromosomal translocations such as PML-RARa, MLL-AF9, and inv(16) have been shown to cause AML as demonstrated using retroviral bone marrow transplantations.¹³⁴⁻¹³⁶ These models were used to collectively illustrate that AML is the consequence of cooperation between at least two classes of mutation, one that impairs hematopoietic differentiation, and a second that confers a proliferative and/or survival advantage. Though these studies have been informative

about the biology of FLT3-ITD leukemia, these retroviral bone marrow transplantation models do not physiologically recreate the levels of oncogene expression. Nonetheless, one of the known mechanisms of disease progression in FLT3-ITD AML is the loss of the FLT3-WT allele by acquired segmental uniparental disomy. In recent years, several groups have generated transgenic mouse models showing FLT3-ITD cooperation with other genetic lesions to cause AML, with a loss of heterozygosity. Specifically, knock-in of both FLT3-ITD and Nup98HoxD13 causes an aggressive AML with loss of heterozygosity.¹³⁷ Several groups have showed that nucleophosmin mutations such as NPM_{1C} and NPMc+ also cooperates with FLT3-ITD to cause AML with loss of heterozygosity.^{138, 139} Zorko *et al* also showed that MLL-PTD cooperates with FLT3-ITD to cause leukemia in a FLT3 dosage dependent effect.¹⁴⁰

Role of developmental signaling pathways in leukemia

Recent studies of developmental signaling pathways in leukemia have also shed some light on mechanisms of disease pathogenesis in leukemia. Several of these developmental pathways that have gone awry give rise to leukemogenesis by regulating the maintenance and self-renewal of leukemia initiating cells. Notably, the Wnt/ β -catenin signaling pathway has been reported to play a significant role in regulating leukemic stem cells in CML as well as AML.^{141, 142} Recently, Scheller *et al* showed that Wnt/ β -catenin signaling can contribute to CML progression to blast crisis and drug resistance by driving self-renewal and myeloproliferation.¹⁴³ In CML, TGF β -FOXO signaling also plays a role in maintaining leukemia initiating cells.¹⁴⁴ On the other hand, while activating mutations of Notch1 have been found in human T-cell ALL, and Notch signaling is also implicated in the pathogenesis of APL, Notch signaling has been identified as a tumor suppressor in AML.^{145, 146} Notch pathway activation inhibits growth of AML by disrupting homeostasis and differentiation of disease initiating cells.^{147, 148} In a similar differentiation-inducing approach, elevated Akt/FOXO signaling found in AML patients, were required to maintain leukemia initiating cells; decreasing FOXO3 can increase myeloid maturation and

induce apoptosis in disease initiating cells.¹⁴⁹ Intriguingly, FLT3-ITD mutations have been reported to cooperate with Wnt signaling in leukemia, and the signal transduction pathways synergize in myeloid transformation.¹⁵⁰ Wnt pathway activity also plays a role in modulating the sensitivity of FLT3 pathway inhibitors, suggesting the clinical significance of understanding the role of developmental signaling pathways in leukemia.¹⁵¹

Interestingly, these developmental signaling pathways have not been shown to overtly initiate malignancy formation. Wnt signaling has been shown to be dispensable for adult HSC maintenance.¹⁵² While Notch signaling may be essential for HSCs during fetal development, and also plays a role in the lymphoid lineage, its inactivation results in a myeloproliferative disease and not AML. Similarly, though Hh signaling has been implicated in both normal adult and embryonic hematopoiesis, the precise role of Hh signaling has been controversial, depending on the stage of development, cell types examined, physiological state and methods of interrogation. Mouse models of Hh modulation have been used to elucidate the role of Hh signaling in the hematopoietic system. Examples include activation of Hh signaling by deletion of *Ptch1* and *Smo* dependent Hh signaling transgenic mouse models. While studies by Dierks *et al* and Zhao *et al* showed that deletion of *Smo* in the mouse affected hematopoiesis, two seminal studies challenged this view in 2009 when they both suggested that *Smoothed* was dispensable for adult hematopoiesis, based on the use of an inducible conditional *Smo* knockout model in adult mice.^{108, 109} A Cre recombinase under the control of the myxovirus-resistance 1 (*Mx1*) gene promoter (Mx1-Cre) when stimulated with poly(I:C), allowed for interferon-inducible *Smo* deletion in the hematopoietic system.¹²⁵ The deletion of *Smo* in adult mice did not affect hematopoiesis. The positive downstream effector of the Hh pathway, *Gli1*, however, did have an effect on hematopoiesis. Merchant *et al* found that while *Gli1* null mice had an expanded LT-HSC compartment, there was impaired myeloid differentiation and defective response to stress in the proliferative progenitor compartments.¹⁵³

Interaction between signaling pathways in cancer development

These studies suggest that while these pathways play a role in maintaining self-renewal in disease initiating cells, their contribution to advance disease and poor prognosis may be in the form of accelerating existing oncogenic lesions by enhancing cross talk between signaling transduction pathways. Recent evidence suggests that activation of Hh signaling in neoplasms may result from the integration of multiple deregulated oncogenic signaling inputs in the final signaling effectors of the pathway, the GLI transcription factors. Growth factors such as platelet-derived growth factor, epidermal growth factor, and insulin-like growth factor 1 increase activation of the Hh pathway by way of activating the PI3K-AKT pathway. Interplay between Hh pathway and NF- κ B, RAS-RAF-MEK, TGF- β , and notch pathways have also been reported in several cancers. An understanding of how major oncogenic pathways interact with each other to drive tumorigenesis will enhance the development of more effective and targeted therapies. We speculate that one of the ways disease progression can occur in leukemia is by cross talk between different signaling pathways. A prominent example would be the evidence of cross talk between the Hh and the EGFR pathways shown in skin cancers. Coactivation of EGFR and Gli1 resulted in the activation of selected GLI target genes. This cooperation also was mediated by the activation of MEK/ERK signaling by EGFR. In FLT3-ITD driven leukemias with upregulated Hh signaling, we show that cross talk between the FLT3 tyrosine kinase signaling pathway and Hh pathway can enhance Stat5 signaling and proliferation. Intriguingly, *JunB* and *Klf6*, both found to be significantly upregulated in SmoM2; FLT3-ITD mice, have also been shown to be implicated in the epithelial-mesenchymal transition in response to TGF- β , a known mechanism of disease progression in solid tumors.¹⁵⁴

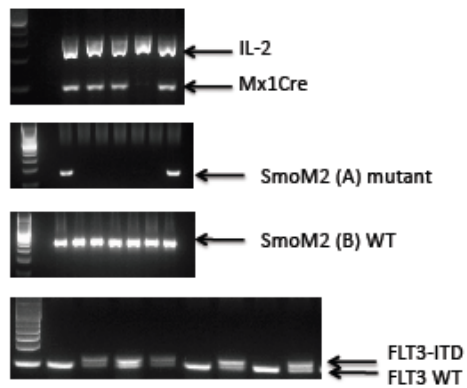
We show in our model that a developmental signaling pathway that has no effect on hematopoiesis on its own can cooperate with FLT3-ITD to cause AML. Disease progression

occurs rapidly when Hh signaling cooperates with and augments FLT3-ITD signaling to enhance proliferation.

Supplementary Figures

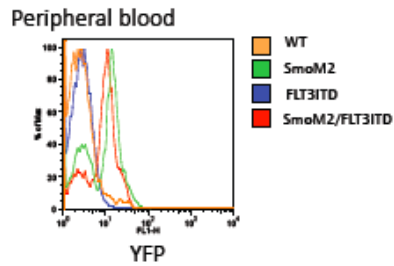
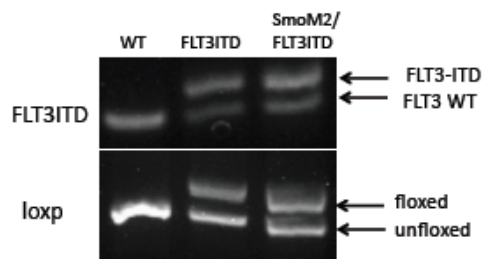
Supplementary Figure 1: Genotyping of transgenic mice by PCR

PCR using specific primers were run using genomic DNA extracted from mouse tail clippings as a template. The PCR product was run on a 3% agarose gel.



Supplementary Figure 2: Verification of excision of transgenes in mouse model

Peripheral blood was obtained via the retro-orbital sinus 3 weeks after the last poly(I:C) injection. Genomic DNA was extracted from peripheral blood and PCR reactions were performed to determine if the FLT3-ITD transgene was successfully excised. Peripheral blood was also lysed by RBC lysis buffer, and analyzed by flow cytometry to detect YFP, as an indication of successful expression of SmoM2.



Supplementary Figure 3: Leukemic infiltration in non-hematopoietic organs

Necropsies were performed on SmoM2; FLT3-ITD animals at the time of their death from leukemia, and the extent of mononuclear and myelocytic cell infiltration was determined. Representative hematoxylin and eosin staining of lung and liver sections are shown.

Table 1. Tissue Involvement in Lymphoproliferative Lesions

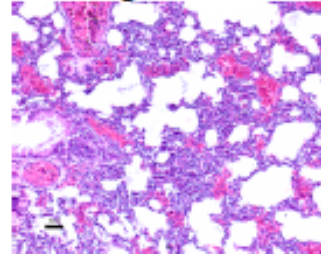
Genotype/I.D.	Tissue/ Organ					
	Spleen	Bone Marrow	Liver	Lung	Kidney	Thymus
BM091209H 12.1 /Dbl Tg	3+	3+	wnl	wnl	1+	(depleted)
BM091209I 12.4/Dbl Tg	2+	1+	wnl	wnl	1+	wnl
BM091209A 17.1/Dbl Tg	3+	3+	3+	3+	1+	(depleted)
BM091209B 19.1/Dbl Tg	3+	3+	1+	2+	wnl	(depleted)
BM091209C 19.3/Dbl Tg	3+	3+	2+	wnl	wnl	(depleted)
BM091209D 19.4/ FLT3 ITD	1+	1+	1+	wnl	wnl	wnl
BM091209E 20.1/SmoM2	wnl	wnl	wnl	wnl	wnl	wnl
BM091209F 20.5/ WT	wnl	wnl	wnl	wnl	wnl	wnl
BM091209G 28.6/SmoM2/ noPIPC	wnl	wnl	wnl	wnl	wnl	wnl

All except 28.6 have Mx1 Cre and were induced with PIPC
Dbl Tg = SmoM2 + FLT3 ITD + Mx1 Cre

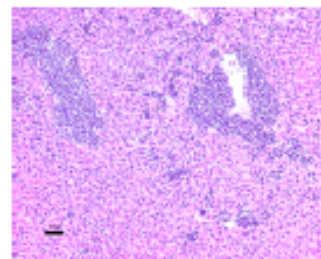
wnl = within normal limits
1+ = mild
2+ = moderate
3+ = marked

Note: Infiltration of liver, lung, and kidney by cells mononuclear/ myelocytic cells

Lung infiltrates

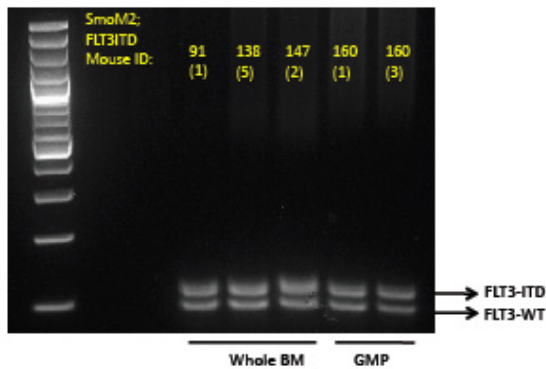


Liver infiltrates



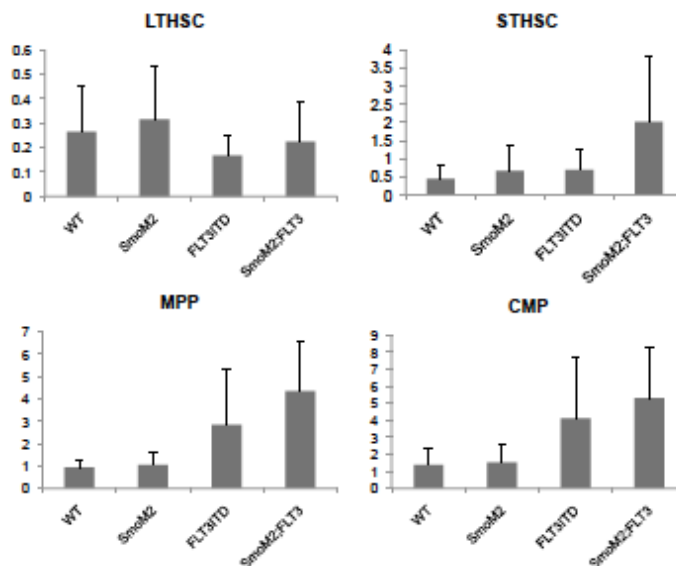
Supplementary Figure 4: No evidence of loss of heterozygosity in diseased mice

DNA was extracted from whole bone marrow as well as sorted GMP cells of SmoM2; FLT3-ITD mice at their time of death from leukemia, and PCR was performed using primers that detect FLT3-ITD and wild-type FLT3. PCR products were visualized on a 3% agarose gel.



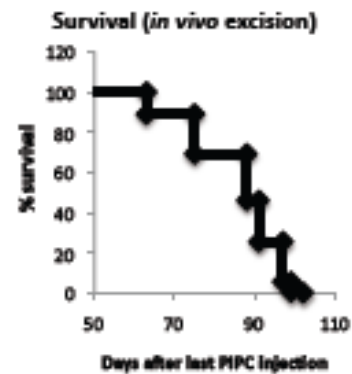
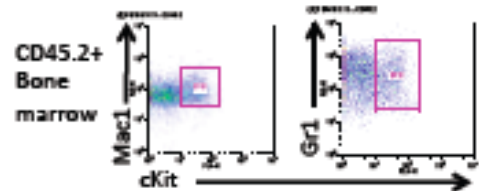
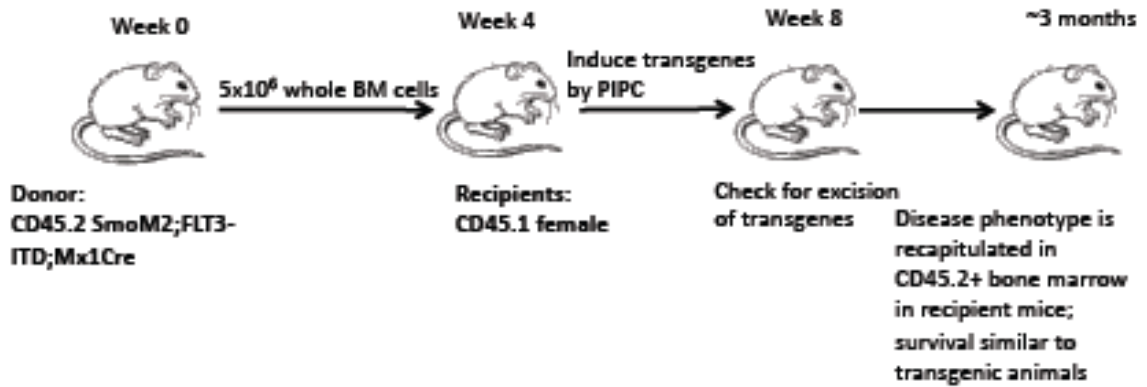
Supplementary Figure 5: No significant differences in primitive HSC frequencies

Whole bone marrow cells from moribund SmoM2; FLT3-ITD animals and their littermate controls were stained with cell surface antibodies and analyzed on the FACS Calibur to determine frequencies of each cell population.



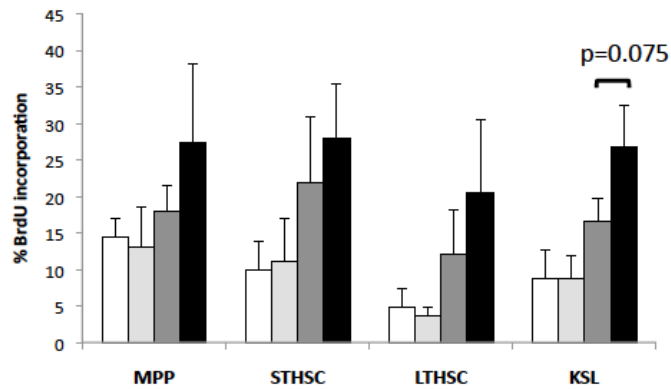
Supplementary Fig 6: Cell autonomous disease model

SmoM2; FLT3-ITD chimeric mice were administered poly(I:C) to induce the SmoM2 and FLT3-ITD transgenes in the CD45.2 population. SmoM2; FLT3-ITD disease phenotype is recapitulated in the CD45.2 population. The SmoM2; FLT3-ITD chimeric animals also die in a similar time period after poly(I:C) administration as seen in the survival curve.



Supplementary Fig 7: BrdU incorporation in HSC compartments

Bar graphs show % of *in vivo* BrdU incorporation in primitive HSC populations. Data are not statistically significant.



CHAPTER 5: POTENTIAL CLINICAL APPLICATIONS

Introduction:

Hedgehog inhibitors

This pivotal association of Hh signaling in oncogenesis suggests that targeting components of the Hh pathway can be an enticing therapeutic strategy in managing cancers. Given its role as a positive effector in the pathway and in several malignancies, Smoothened has emerged to be a prominent target. Indeed, the discovery of cyclopamine, a natural compound that can inhibit Hh signaling by direct binding to Smo, provides evidence that the Hh pathway is susceptible to inhibition by exogenous small molecules, especially at the level of Smoothened.^{155, 156} However, due to the limited potency and poor oral solubility of cyclopamine, it has not been used in the clinic.¹⁵⁷ Nevertheless, since the discovery of cyclopamine, several more potent and specific Smo inhibitors have been developed and are currently in clinical trials for the treatment of a variety of cancers¹⁵⁸. Vismodegib, an orally active Smo antagonist used to treat advanced basal cell carcinoma (BCC), has become the first synthetic Smo inhibitor to be approved by the FDA in 2012.¹⁵⁹ Vismodegib was also used in a patient resulting in regression of advanced medulloblastoma.¹⁶⁰ Despite these successes, a subsequent study reported that other solid tumors did not respond to Vismodegib.¹⁶¹ Vismodegib did not show sufficient efficacy in metastatic colorectal cancer and advanced ovarian cancer in Phase II trials.^{162, 163} Saridegib, another Smo inhibitor developed by Infinity, also failed to show an overall survival benefit in metastatic pancreatic cancer.¹⁶⁴ The lack of positive results could be explained by the fact that these patients had very advanced, multiply treated malignancies, and were monitored over a relatively short follow-up time. However, these failures also stem from a lack of understanding of how Hh signaling is contributing to disease in these other cancers. In basal cell carcinoma, Hh signaling has been shown to be the cause of the disease, however in other cancers, the activation of Hh signaling may be one of several drivers of disease pathogenesis.¹⁵⁷ Furthermore, Hh signaling appears to have distinct mechanisms of action in different tumor types and environments.

Ligand-independent activation of the pathway drives malignancies such as BCC and medulloblastoma through cell intrinsic mutations in PTCH and SMO, as shown using mouse models.^{101, 102} In particular, recurrent mutations in SMO include L412F, R562Q (*SMO* M1) and W535L (*SMO* M2), and functional studies have demonstrated that such mutations, which result in aberrant activation of Hh signaling, promote tumorigenesis.⁹⁹ Hh signaling can also occur in an autocrine or paracrine manner between tumor cells and its microenvironment by ligand overexpression, such as in glioblastoma, lymphoma and pancreatic cancer.¹⁶⁵ Evidence supporting ligand dependent paracrine signaling has accumulated from studies in human tumor xenograft mouse models of pancreatic and colorectal cancer that highly express Hh ligand, and increased Hh target gene expression is observed in the infiltrating mouse stromal cells.¹⁶⁶ Additionally, in reverse paracrine signaling, another ligand-dependent mechanism, the bone marrow stroma in cancers such as lymphomas and multiple myelomas is the source of Hh ligand, which then activates Hh signaling in the tumor.¹⁶⁷ Nevertheless, the precise molecular mechanisms of how Hedgehog pathway stimulation positively regulates tumor growth are not completely understood, given an appreciation of the varying contextual roles of the signaling pathway. Furthermore, the role of Hh inhibitors in cancers where components of the pathway are not mutated is unclear. New mutations in Smo can also be acquired, resulting in resistance to Smo inhibitors such as the case of Vismodegib in the treatment of medulloblastoma.¹⁶⁸ Nevertheless, despite a lack of mechanistic understanding of Hh signaling in cancer, these Smo inhibitors have been fairly well tolerated and have little to no hematologic toxicities.¹⁶⁹ Most of the existing pre-clinical studies using Smo inhibitors show moderate tumor growth inhibition in xenograft and transgenic mouse models, suggesting that Hh inhibition alone may not be sufficient, and hence combinations with other agents may be necessary to achieve maximum benefit.

FLT3 inhibitors

The impressive response of chronic myelogenous leukemia patients to BCR-ABL tyrosine kinase inhibitors (TKI) generated enthusiasm for molecularly targeted therapies in malignancies dependent on constitutively activated kinase signaling. FLT3 inhibitors have begun to show promise in clinical trials, and there is currently great enthusiasm that small molecule tyrosine kinase inhibitors will transform the care of a large number of AML patients.¹⁷⁰ However, similarly to the development of resistance to imatinib from acquisition of point mutations in BCR-ABL, mutations in FLT3-ITD have been observed in AML patients treated with TKIs.¹⁷¹ The development of these FLT3 inhibitors has been hampered by weak and transient inhibition. Furthermore, patients have developed resistance to these inhibitors, largely by acquiring other tyrosine kinase mutations.¹⁷²

Sorafenib, a small molecule inhibitor that targets a number of serine/threonine and receptor tyrosine kinases is the first oral agent approved for the clinical treatment of advanced renal and hepatocellular cancers.^{173, 174} Sorafenib has been reported to directly inhibit mutant FLT3 in the treatment of AML, and is currently used to manage the disease in patients.¹⁷⁵

Method:Cell culture and drug treatment

Human MV411 and Molm14 cell lines harboring the FLT3-ITD mutation were cultured in RPMI media supplemented with 10% FBS, 0.5% L-Glutamine and 0.5% Pen/Strep. Both IPI 926 (Infinity Pharmaceuticals) and Sorafenib (LC laboratories) were dissolved in 100% DMSO (Sigma). Sorafenib was prepared as 10uM stock solutions in RPMI with 0.1% DMSO and stored at -80⁰C. The cell lines were treated with either 2.5uM of IPI 926 or 1nM of Sorafenib for MV411 cells and 3nM for Molm14 cells, or a combination of both. Recombinant Shh ligand was purchased from R&D Systems and used at a concentration of 2ug/ml. Cells were seeded at 100,000 cells/ml and cultured in the 37⁰C incubator for 3 days with the inhibitors. Viable cell counts were obtained every 24 hours by the Trypan blue exclusion assay.

Cell cycle analysis

MV411 and Molm14 cells were washed with PBS following drug treatment. The cell pellets were resuspended in PBS containing 0.6% NP40 (Sigma), 40ug propidium iodide and 2ug/ml RNase (Roche). The mixture was vortexed and kept on ice for 15 minutes, following which cell cycle analysis was performed using the FACS Calibur (BD Biosciences) and analyzed using FlowJo software (Flow Jo).

Apoptosis assay

MV411 and Molm14 cells were washed with PBS following 3 days of drug treatment. The cell pellets were resuspended in 100ul of 1X Binding buffer, and stained with AnnexinV-FITC antibody and PI according to manufacturer's protocol (BD Biosciences). Following staining, the

cells were immediately analyzed to determine the percentage of AnnexinV⁺ PI⁺ dead cells by flow cytometry on the FACS Calibur (BD Biosciences).

Colony formation

MV411 and Molm14 cells were treated with IPI 926, Sorafenib and a combination of both for 3 days. At day 3 after drug treatment, 500 cells from each treatment group was plated onto semi-solid methycellulose media and incubated at 37°C. Total colony counts were obtained 7 days after plating. Data is representative of 3 independent experiments.

In vivo drug treatment

IPI 926 (Infinity Pharmaceuticals) was formulated in a 5% β -cyclodextrin (Sigma) solution with deionized H₂O according to manufacturer's protocol. Mice were given 20mg/kg of IPI 926 or the same volume of 5% β -cyclodextrin solution once a day for 16 days by oral gavage.

Sorafenib (Bayer) was formulated in 100% DMSO (Sigma). Mice were given 10mg/kg of Sorafenib or vehicle once a day for 16 days by oral gavage. Vehicle solution consisted of 30% (w/v) Cremophor EL, 30% (w/v) PEG 400, 10% ethanol, and 10% glucose (all Sigma-Aldrich). Mice were also given the combined treatment of Sorafenib and IPI 926. The vehicle treated group received both 5% β -cyclodextrin solution as well as the vehicle solution for Sorafenib.

Statistics

Log-rank (Mantel-Cox) test p-values for the Kaplan-Meier survival curves were generated using GraphPad Prism. All other p values were calculated using an unpaired two-tail Student's t test, unless otherwise specified. All data were collected from at least 3 independent experiments, and presented as the mean \pm standard deviation.

Results:

Sorafenib and IPI 926 synergistically decrease leukemic cell growth *in vitro* and *in vivo*.

In adult AML, activating mutations of the FLT3 tyrosine kinase in the form of internal tandem duplication insertions in the juxtamembrane domain or point mutations in the kinase domain are the most commonly found mutation. FLT3-ITD mutations in particular are found in approximately 30% of de novo AML cases. These patients with the mutation also have a poor prognosis, and are highly prone to relapse. Therefore, there is a lot of interest in generating and using inhibitors that target the FLT3 signaling pathway. However, the challenges to effectively using these inhibitors include resistance to tyrosine kinase inhibitors, emergence of kinase domain mutations, as well as dependence on other pathways. Combining the use of these FLT3 inhibitors with other treatment modalities and other pathway inhibitors may overcome some of these challenges. To further investigate the functional relevance of Hedgehog signaling in human FLT3-ITD AML, we pharmacologically inhibit both the Hedgehog and FLT3 signaling pathways in human FLT3-ITD AML cell lines. Sorafenib has been shown to have significant activity against FLT3-ITD in human cells.¹⁷⁵ IPI 926 is a semi-synthetic small molecule antagonist of Smoothened currently in Phase 1 and 2 clinical trials. We treated Molm14 and MV411 human leukemia cell lines, of which both harbor the FLT3-ITD mutation, with both Sorafenib and IPI 926. Interestingly, we observed that IPI 926 does not have any effect on the growth of the cancer cells. While Sorafenib alone decreases cell growth over time, the combination of Sorafenib and IPI 926 further reduces cell growth in both the cell lines (Figure 5.1A). This effect is specific given that Sorafenib does not impact the growth of HL60 cells, which have wild-type FLT3. To determine if increased Hedgehog signaling can override the effects of Sorafenib, we treated the cells with recombinant Sonic Hedgehog (Shh) ligand. In the presence of the Shh ligand,

Sorafenib was not as effective in decreasing cell growth (Figure 5.1B), suggesting that the effect is specific to Hh signaling.

To investigate the mechanism of decreased cell growth and viability with combined Sorafenib and IPI 926 treatment, we examined apoptosis by AnnexinV and Propidium Iodide (PI) staining as well as cell cycle progression by PI staining in Molm14 cells. There was a trend towards increasing AnnexinV⁺ PI⁺ cells when Sorafenib treatment is combined with IPI 926 (Figure 5.1C), as well as a significant difference in cell cycle progression. Sorafenib together with IPI 926 caused more cells to arrest in G1 phase of cell cycle progression compared to just Sorafenib alone (Figure 5.1D). The effects of combining Sorafenib and IPI 926 appear to be synergistic (Supplementary Fig 8). These experiments were also performed using MV411 cells, which showed a similar result (data not shown). There was also a significant decrease in the clonogenic growth of cells treated with both Sorafenib and IPI 926 compared to Sorafenib alone (Figure 5.1E). These results support the overall decrease in cell viability, and show that simultaneously inhibiting both the FLT3 and Hh signaling pathways in human FLT3-ITD AML cell lines can reduce leukemic growth.

Figure 5.1. Sorafenib and IPI 926 synergistically decrease leukemic cell growth *in vitro*

(A) Viable cell counts using Trypan Blue assay, of Molm14 and MV411 cell lines treated with IPI 926 and Sorafenib, data from at least 4 independent experiments * $p < 0.05$

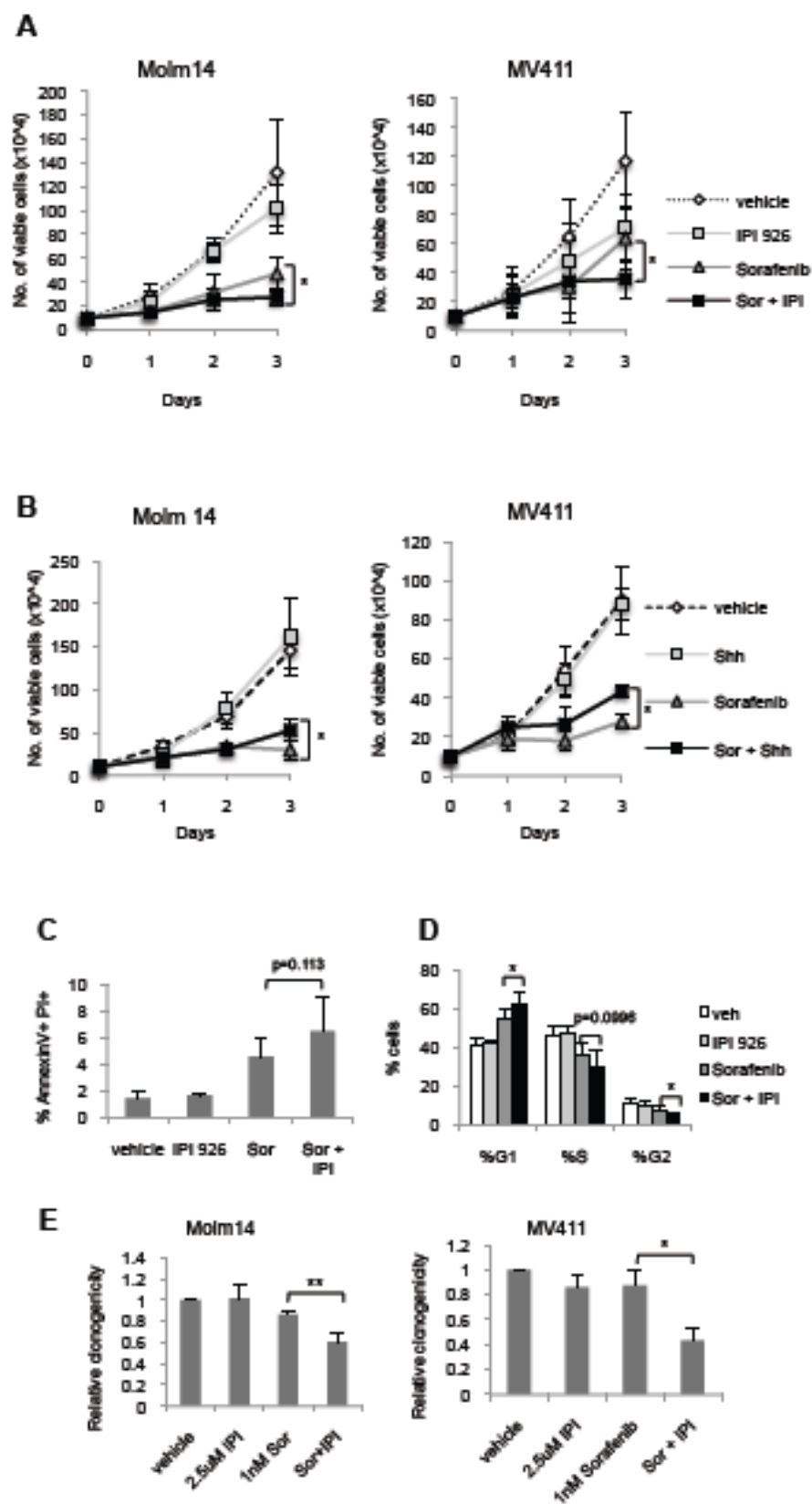
(B) Viable cell counts of Molm14 and MV411 cell lines treated with recombinant Shh ligand and Sorafenib, data from at least 3 independent experiments * $p < 0.05$

(C) Bar graphs showing percentage of AnnexinV+ PI+ cells in Molm14 cells treated with IPI 926 and Sorafenib, data from 3 independent experiments.

(D) Bar graphs showing percentage of cells in G1, S and G2 phases of cell cycle progression by PI staining in Molm14 cells treated with IPI 926 and Sorafenib, data from 3 independent experiments. * $p < 0.05$

(E) Bar graphs showing relative clonogenicity of MV411 and Molm14 cells treated with IPI 926 and Sorafenib for 3 days and plated in methycellulose, data from at least 3 independent experiments. * $p < 0.05$, ** $p < 0.01$

Figure 5.1



To further test the efficacy of combining these inhibitors *in vivo*, we generated chimeric CD45.1 mice engrafted with CD45.2 SmoM2; FLT3-ITD; Mx1Cre bone marrow, administered poly(I:C) to induce the transgenes, and delivered the drugs by oral gavage 3 months after induction of the transgenes (Supplementary Fig 6). We confirmed that the transgenes were excised in these chimeric mice by PCR and flow cytometry, and also confirmed that they recapitulated the disease phenotype in CD45.2 bone marrow by flow cytometry. These mice were treated with the drugs once a day consecutively for 16 days. Immediately post-treatment, mice receiving both Sorafenib and IPI 926 showed a reduction in spleen sizes compared to the other treatment groups (Figure 5.2A). Mice treated with both Sorafenib and IPI 926 for 16 days also had better overall survival compared to just Sorafenib or IPI 926 treatment alone (Figure 5.2B), showing that combinatorial therapy by inhibiting both pathways simultaneously can rescue the animals from death from FLT3-ITD AML.

Combinatorial therapy with Sorafenib and IPI 926 appear to reduce the growth of FLT3-ITD leukemia cells both *in vitro* and *in vivo*. We provide rationale and evidence for combining the inhibitors in the clinic, as well as evidence that a developmental signaling pathway such as Hedgehog can be an accelerator of disease progression.

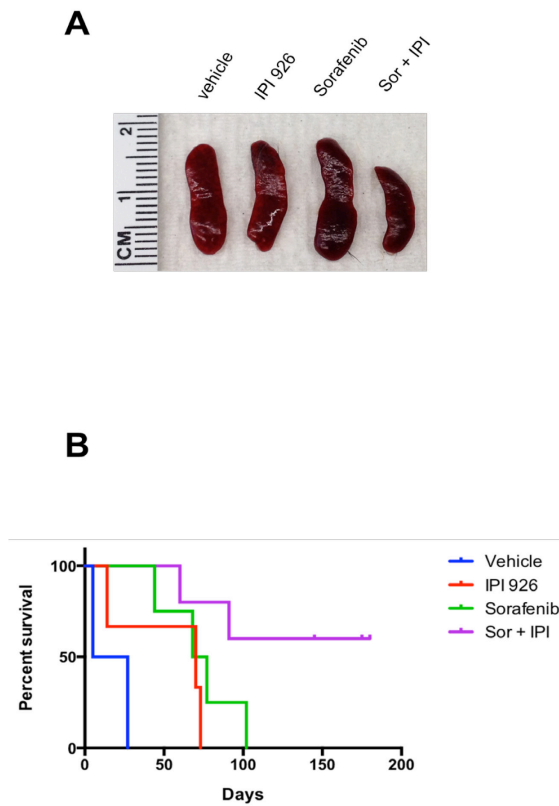
Figure 5.2 Combined Sorafenib and IPI 926 treatment increases overall survival *in vivo*

(A) Spleen sizes of drug treated animals immediately after 16 days of treatment

(B) Kaplan Meier survival curve of animals treated with Hh and FLT3 pathway inhibitors.

Mantel-Cox Log-rank P value <0.001

Figure 5.2



Discussion:

Limitations of FLT3 inhibitors

Although FLT3 signaling remains a great therapeutic target, especially with FLT3-ITD being the most common mutation, responses are transient and leukemia progression invariably occurs. Single base mutations at the FLT3 tyrosine kinase domain (TKD) also leads to a gain of function, although its prognostic significance is less well defined because of its rarity. There is compelling evidence that leukemia clones carrying both ITD and TKD mutations appear when resistance to FLT3 inhibitors occurs. The FLT3 signaling pathway might have become refractory to inhibition or alternative survival pathways could have been activated in the leukemic cells. Furthermore, FLT3-ITD⁺ cells might belong to a subclone in the leukemic cell population and new clones might emerge during leukemia progression and relapse. Interestingly, the emergence of double ITD and TKD mutants can be recapitulated in vitro when FLT3-ITD⁺ leukemia cell lines are treated with mutagens and FLT3 inhibitors. Furthermore, murine xenotransplantation models also suggest that, in some cases, the FLT3-ITD and TKD double mutants actually exist in minute amounts before treatment with FLT3 inhibitors, expand under the selection pressure of FLT3 inhibition and become the predominant resistant clone(s) during the drug-refractory phase. On the basis of this model of clonal evolution, a multipronged strategy using more potent FLT3 inhibitors, and a combinatorial approach targeting both FLT3-dependent and FLT3-independent pathways, will be needed to improve treatment outcome.

Limitations of Smo inhibitors

The use of Hh inhibitors together with FLT3 inhibitors could potentially benefit patients with advance or relapsed FLT3-ITD AML, especially if inhibiting Hh has no effect on normal hematopoiesis. Nevertheless, as canonical Smo function is associated with the primary cilium, and hematologic cells have been thought of to lack this cellular protrusion, this could argue

against the case of using Smo inhibitors in myeloid leukemias. There is also increasing evidence of Smo-independent activation of Gli transcription activity by signaling pathways such as the RAS, MAPK and PI3K/AKT pathways. Also, although Sorafenib works more precisely to target FLT3-ITD compared to FLT3-WT, it also targets a variety of other kinases that could activate downstream Hh activity. Nevertheless, despite the approval of Vismodegib by the FDA for advanced basal cell carcinoma, and the promising clinical results for Smo inhibitors in medulloblastoma, the proof of concept in Hh ligand-dependent tumors in the absence of genetic alterations remains to be determined. Existing pre-clinical data has showed modest tumor growth inhibition in cancer xenograft models and genetically engineered mouse models, thus Smo inhibition alone may not provide sufficient efficacy in these cancers, and combinations with other agents might be needed to achieve maximal benefit.

Evidence for combinatorial therapy

One of the most extensively studied combination partners for Smo inhibitors is gemcitabine for the treatment of pancreatic cancer. The single-agent activity of Smo inhibitors on primary tumor growth in pancreatic cancer xenograft models is modest at best and seems to be mediated primarily through stromal pathway inhibition.¹⁶⁶ Recently, in a genetically engineered mouse model of pancreatic cancer, it was proposed that saridegib sensitizes tumors to gemcitabine treatment through depletion of the tumor stroma.¹⁷⁶ Moreover, the combination of cyclopamine or saridegib with gemcitabine was active in an orthotopic metastatic model of pancreatic cancer, putatively through combined cytoreductive and cancer stem cell mediated effects of gemcitabine and saridegib, respectively.^{177, 178} However, results from a phase 2 trial in patients with pancreatic cancer failed to show a benefit over single agent gemcitabine, highlighting the challenges of testing Smo inhibitors in patients without known driver mutations.

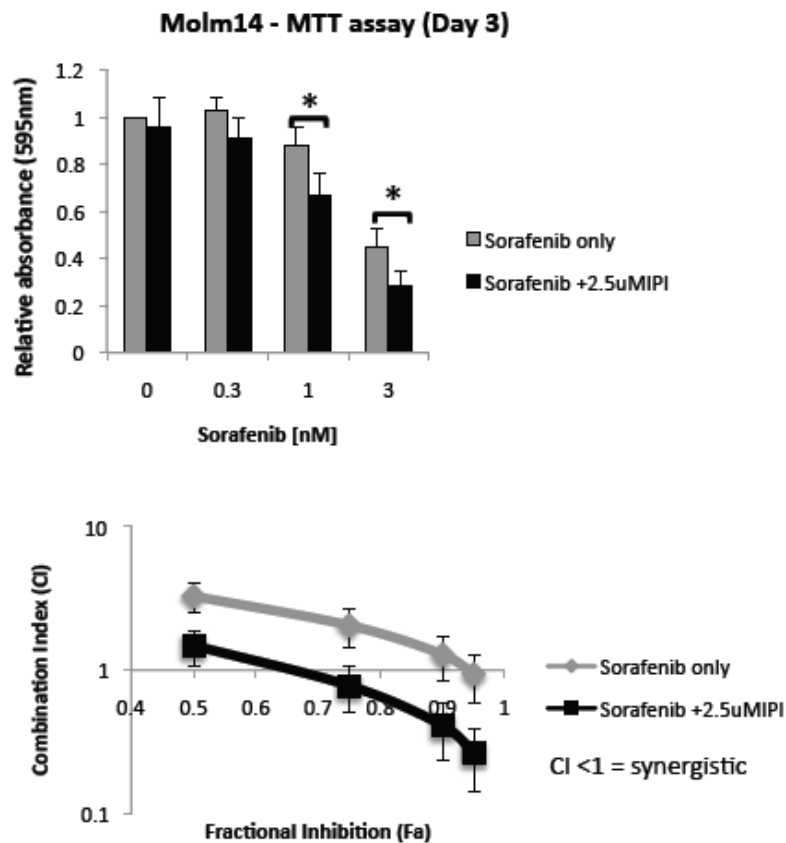
Additional support for combination strategies also stems from the growing body of preclinical data showing evidence of noncanonical activation of Hh signaling in tumors mediated through the PI3K-AKT pathway.¹⁷⁹ Furthermore, the MEK-ERK-JUN signaling axis appears to be the driver for the crosstalk between the Hh and epidermal growth factor receptor (EGFR) pathways.¹⁸⁰ The effects of combining EGFR and SMO inhibitors on tumors have been described in several *in vitro* preclinical models, including basal cell carcinoma, prostate cancer and glioblastoma.^{181, 182} The MEK-ERK pathway has also been shown to link mutant *KRAS* to Hh pathway activation in pancreatic cancer and melanoma.^{183, 184} These data highlight the potential of new combination opportunities for Smo inhibitors in cancers with unmet treatment needs. Nonetheless, we have provided pre-clinical rationale using both *in vitro* and *in vivo* studies for potentially combining both inhibitors as a therapeutic strategy for patients with advanced or relapsed FLT3-ITD AML.

Supplementary Figures

Supplementary Fig 8: Synergistic effects of Sorafenib + IPI 926 combined therapy

Bar graphs show relative absorbance from MTT assay. n=3. *P<0.05

Combination index of drug treatment is calculated to show synergistic interaction of Sorafenib and IPI 926 on Molm14 cells. A similar effect was also observed using MV411 cells (data not shown).



CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions:

In this dissertation, methods to study hematopoiesis in both the normal and malignant state have been described and applied. Starting with normal hematopoiesis, we studied a unique but important way that this normal state can be insulted, and in turn, discover a potential therapeutic strategy for mitigating these damages to bone marrow cells. Much is known about the damaging effects radiation, however the major unanswered question in radiation epidemiology, is not whether radiation causes cancer, but what the level of risk is following low dose rate exposures.¹⁸⁵ As seen from the recent Fukushima nuclear reactors accident, low dose exposures of radiation received over time are likely to impact large communities, but the risks are still unknown. We showed that protracted low dose-rate radiation exposure affects hematopoiesis, and that chloroquine can be a potential prophylactic treatment by enhancing engraftment of myeloid progenitors to maintain hematopoiesis. Our whole animal model can also be used to further study the effects of protracted low dose-rate radiation on other organ systems.

Learning about how normal hematopoiesis is perturbed can shed light on how malignant hematopoiesis arise and in turn, how to interrupt the malignant transformation process. To better understand disease progression, we generated a novel mouse model of myeloid disease progression and showed that Hedgehog signaling, a developmental signaling pathway that has no impact on normal adult hematopoiesis has a role to play in myeloid disease acceleration initiated by the FLT3-ITD oncogene. Importantly, there are small molecule inhibitors against both the Hedgehog and FLT3 signaling pathways; we showed that combinatorial therapy using these inhibitors could reduce leukemic growth and improve overall survival, suggesting a viable therapeutic strategy for patients with advanced FLT3-ITD AML.

Future directions:

Future studies could include an investigation into the mechanism of chloroquine protection against low dose-rate radiation. It is likely that chloroquine acts by multiple mechanisms *in vivo*, and it is possible that additional protective functions including those mediated by the bone marrow microenvironment also contribute to its activity. It is also conceivable that the survival benefit associated with chloroquine may be associated with an increased accumulation of genetic alterations and mutations. Therefore, further investigation of chloroquine's action that include both ATM-mediated and independent processes is warranted. A potential side effect of chloroquine in combination with radiation may be cutaneous desquamation has been reported.¹⁸⁶ We did not observe skin reactions in the mice treated with chloroquine and low dose-rate radiation. Nevertheless, given its tolerability and low cost, chloroquine may serve as a readily accessible agent drug for protection from LDR radiation injury. Our study using mice could also be expanded to include larger animals such as dogs, pigs and chimpanzees. Furthermore, the efficacy of chloroquine in radioprotection after radiation exposure should also be examined.

From our pre-clinical studies with Hh and FLT3 inhibitors, a clinical trial involving combinatorial therapy of Sorafenib and IPI 926 in patients with advanced or relapsed FLT3-ITD AML could be initiated. Other FLT3 inhibitors currently in development can also be tested in combination with IPI 926. The rapid advancement of Smo inhibitors into clinical development has provided exciting results in mutation-driven tumors with activated Hh signaling and led to the approval of vismodegib as a first-in-class treatment for advanced BCC, but it has also revealed that the clinical application of single-agent Smo inhibitors might not be as broad as was initially expected. Tumors that have shown clinically meaningful responses are either very rare (such as medulloblastoma) or rarely require systemic therapy (such as BCC). BCC is almost exclusively dependent on Hh signaling, and not surprisingly, these tumors are exquisitely sensitive to Smo

inhibition. Nonetheless, a number of questions remain regarding the optimal dosing schedule, the optimal treatment duration and whether a complete cure is achievable.

Notably, all these negative trials included unselected patients. Given the encouraging preliminary results in selected patients with Hh-activated medulloblastoma, it is important to invest more effort in the exploration of the evolving knowledge of crosstalk between Hh signaling and other oncogenic pathways. Given the emerging complexity of Hh pathway activation in cancer, it will be important to differentiate Smo-dependent from Smo-independent canonical or noncanonical pathway activation. A detailed understanding of the mechanism leading to GLI activation in each tumor will allow for selection of the appropriate Hh pathway inhibitor and, in cases where crosstalk between Hh signaling and other oncogenic pathways exist, the optimal combination partner. Furthermore, mouse models increasingly hold tremendous potential for cancer therapy, given the sophistication of genetic manipulation techniques as well as commercial availability of these murine systems, and can greatly facilitate translating research findings into the clinics.¹⁸⁷ Further work on the potential interaction of FLT3 tyrosine signaling pathways and other signaling pathways could also be explored.

Finally, the potential of an epithelial-mesenchymal-transition (EMT) like program responsible for transformation in leukemogenesis could be further explored. Although the EMT theory has been evidenced from solid tumors that have the ability to metastasize, the underlying core machinery that drives this transition, and not the overt phenotype, may fuel the transformation of cancers in general.¹⁸⁸ Hematopoiesis and its malignancies can be a great system to use to investigate this.

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Curriculum Vitae

Yiting Lim

Contact information

Address:
1650 Orleans Street
The Bunting Blaustein Cancer Research Building
Room 230
Baltimore MD 21287
USA

Tel: +1 (410) 207-8218
Email: yiting.yl@gmail.com

Date and place of birth: Feb 15th, 1983. Johor Bahru, Malaysia.

Education

PhD candidate, Johns Hopkins University School of Medicine,
Pathobiology graduate program (Thesis advisor: William Matsui, M.D.) 2007 – 2014

B.S. (with honors), Life Science (Molecular and Cell Biology), National
University of Singapore; 2007
Minor in Technopreneurship, University of Pennsylvania/National
University of Singapore Overseas College Program

Research presentations

Poster presentation at 2 nd HEALING International meeting for Hedgehog-GLI signaling, Arolla, Switzerland.	2013
Poster presentation at American Society of Hematology annual meeting	2010
Poster presentation at Pathobiology graduate program annual retreat	2009, 2010, 2012
Poster presentation at Pathology Young Investigators' Day	2010, 2011, 2012, 2013
Poster presentation at SKCCC fellows research day	2009, 2010, 2012

Awards and honors

Excellence in Translation Research Award, Pathology Young Investigators Day	2012
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Certificate of Excellence (top 5% reviewers for Laboratory Investigation)	2011
Certificate of Meritorious Service, Editorial Intern, Laboratory Investigation (2010-2011)	2011
Travel award, American Society of Hematology	2010
Best poster (year 4 and above) at Pathobiology graduate program annual retreat	2010
Caltech summer undergraduate research fellowship, HHMI SURF fellow	2006
Academic scholarship, Association of South East Asian Nations (ASEAN) scholarship	1996-2006

Publications

Toshihiko Tanno, **Yiting Lim**, Qiuju Wang, Marta Chesi, P. Leif Bergsagel, Geoff Matthews, Ricky W. Johnstone, Suzanne Sebald, Se-Jin Lee, Ivan Borrello, Carol Ann Huff, William Matsui.

GDF15 enhances the clonogenic growth of multiple myeloma cells
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Yiting Lim, Mohammad Hedayati, Akil Merchant, Yonggang Zhang, Michael B. Kastan, William Matsui, Theodore Dewese.

Chloroquine Improves Hematopoietic Recovery and Survival following Lethal Low Dose-Rate Radiation.
Int J Radiat Oncol Biol Phys 2012, Nov 1;84(3):800-6

Yiting Lim, William Matsui.

Hedgehog signaling in hematopoiesis.
Crit Rev Eukaryot Gene Expr. 2010 20(2):129-139.

Jeffrey G. Marblestone, Suzanne C. Edavettal, **Yiting Lim**, Peter Lim, Xun Zuo, and Tauseef R. Butt.

Comparison of SUMO fusion technology with traditional gene fusion systems: Enhanced expression and solubility with SUMO.
Protein Sci 2006 15: 182-189

Zeshaan A. Rasheed, NV Rajeshkumar, Clinton Jung, Theodore Ewachiw, Ally Huang, Michelle Rudek, Ming Zhao, Ross McMillan, Vesselin Penchev, **Yiting Lim**, Gabriel Ghiaur, Ana DeJesus-Acosta, Daniel Laheru, James Eshleman, Michael Goggins, Karen McGovern, Margaret Read, Vito Palombella, Anirban Maitra, Farhad Parhami, William Matsui
Liver X receptor activation represses Gli1 and enhances gemcitabine sensitivity in pancreatic adenocarcinoma (manuscript in preparation)

Mentoring experience

Sarah Foerster Masters student, German Cancer Research Center (DKFZ) Heidelberg, University of Heidelberg	Aug 2012 – Jan 2013
Nina Hosmane PhD candidate, lab rotation, Cellular and Molecular Medicine program, Johns Hopkins University School of Medicine	2012
Ross McMillan MD-PhD candidate, lab rotation, Cellular and Molecular Medicine program, Johns Hopkins University School of Medicine	2011
Angel Gonzalez Undergraduate, Johns Hopkins University	2009-2010
Participated in annual Johns Hopkins Community Science day for elementary/middle school students	2009-2013

Others

Attended 2 nd HEALING International Summer School on Hedgehog-GLI signaling, Arolla, Switzerland	2013
Undergraduate research at Genome Institute of Singapore, Agency for Science, Technology and Research (A*STAR), Singapore. Laboratory of Lawrence W. Stanton, PhD.	2005-2007
Summer undergraduate research program at California Institute of Technology. Laboratory of David J. Anderson, PhD.	2006
Research internship at LifeSensors Inc. (Malvern, PA). Founder: Tauseef R. Butt, PhD.	2004-2005